TRANSMITTAL OF APPEAL BRIEF (Large Entity)

Docket No.
MSU 4.1-672

In Re Application Of: **Muraleedharan G. Nair, Yanjun Zhang and Shaiju K. Vareed**

Application No.	Filing Date	Examiner	Customer No.	Group Art Unit	Confirmation No.
10/725,214	December 1, 2003	Michele C. Flood	21036	1655	4443

Invention: **METHOD FOR INHIBITING CANCER CELLS**

COMMISSIONER FOR PATENTS:

Transmitted herewith is the Appeal Brief in this application, with respect to the Notice of Appeal filed on:
April 17, 2007

The fee for filing this Appeal Brief is: **$500.00**

☒ A check in the amount of the fee is enclosed.

☐ The Director has already been authorized to charge fees in this application to a Deposit Account.

☒ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. 13-0610. I have enclosed a duplicate copy of this sheet.

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Signature

Dated: **June 1, 2007**

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I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to "Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450" [37 CFR 1.8(a)] on
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appln. No.	: 10/725,214	Confirmation No. 4443
Applicants	: Muraleedharan G. Nair, Yanjun Zhang and Shaiju K. Vareed	
Filed	: December 1, 2003	
Title	: METHOD FOR INHIBITING CANCER CELLS	
TC/A.U.	: 1655	
Examiner	: Michele C. Flood	
Docket No.	: MSU 4.1-672	
Customer No.	: 21036	

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Alexandria, VA 22313-1450

BRIEF UNDER 37 C.F.R. § 41.37

Sir:

This is an appeal from a final rejection in the above entitled application. The claims on appeal are set forth as Claims Appendix. An oral hearing will be requested. Enclosed is the fee due upon filing of the Brief.

(1) Real Party in Interest

The real party in interest is the Board of Trustees operating Michigan State University, East Lansing, Michigan, a constitutional corporation of the State of Michigan, which is the assignee of the above entitled application.

(2) Related Appeals and Interferences

This application is a continuation-in-part application of U.S. Serial No. 09/776,527 (U.S. Patent No. 6,656,914), filed February 2, 2001, which is a continuation of U.S. Serial No. 09/494,077, filed January 28, 2000, now abandoned. There are no pending related appeals and interferences.

(3) Status of Claims

Claims 1 and 5-10 are pending in the application. Claims 8-10 were withdrawn from consideration. Claims 2-4 were cancelled during prosecution of the application. No claims have been allowed. Claims 1 and 5-7 remain pending in the application and are presently on appeal.

(4) Status of Amendments

An Amendment Under 37 C.F.R. §1.116 was filed on March 9, 2007 amending Claims 1 and 7. The amendment was not entered. An Amendment Under 37 C.F.R. §1.116 was filed on April 17, 2007 amending Claim 7 to overcome the objection to Claim 7, as suggested by the Examiner in the Final Office Action. The amendment was entered.

(5) Summary of Claimed Subject Matter

The claimed subject matter in independent Claim 1 is a method for *in vivo* suppression in a mammal of multiplicity in the stomach, colon and in both the stomach and colon of cancer cells which comprises: providing an effective amount of a composition which consists essentially of malvidin as an active ingredient to the mammal so as to suppress the multiplicity of the cells.

Support for this independent claim is found in paragraph [0009]-[0010] on page 3 of the specification. Support for malvidin as an inhibitor of

stomach and colon cancer cell lines is found in paragraph [0043]-[0044] of the specification and in Figure 7 where the viability of colon (HCT-116) and stomach (AGS) cells decreases in the presence of 100 ppm and 200 ppm malvidin. Support for a composition which consists essentially of malvidin is provided in paragraph [0032], where it states that the anthocyanidins (such as malvidin) are commercially available and can be isolated from fruits and vegetables.

(6) Grounds of Rejection to Be Reviewed on Appeal

(A.) Claims 1 and 5-7 were rejected under 35 U.S.C. §112, first paragraph, because the specification, while being enabling for an *in vitro* method for inhibiting the proliferation of colon cancer cells and stomach cancer cells, does not reasonably provide enablement for a method for *in vivo* inhibition in a mammal.

(7) Argument

The present application is related to U.S. Patent No. 6,656,914, which is directed to a method for suppressing adenoma multiplicity in a mammal with an anthocyanin or cyanidin. Anthocyanins are compounds that impart color in berries. Anthocyanidins, such as malvidin, are aglycones of anthocyanins. These compounds are present in fruits and are very non-toxic. (Specification: paragraphs [0011], [0016], [0017], [0043] on pages 3, 4 and 15). The presently claimed method utilizes malvidin to suppress a multiplicity of cancer cells in the stomach, colon and in both the stomach and colon of a mammal.

(A.) Claims 1 and 5-7 were rejected under 35 U.S.C. §112, first paragraph, as not being enabled by the specification. The specification, according to the rejection, while being enabling for an *in vitro* method for inhibiting the proliferation of colon cancer cells and stomach cancer cells, does not reasonably provide enablement for a method for *in vivo* inhibition in a mammal.

There are many factors to be considered when determining whether there is sufficient evidence to support a determination that a disclosure does not satisfy the enablement requirement and whether any necessary experimentation is "undue." These factors include, but are not limited to:

(A) The breadth of the claims;

(B) The nature of the invention;

(C) The state of the prior art;

(D) The level of one of ordinary skill;

(E) The level of predictability in the art;

(F) The amount of direction provided by the inventor;

(G) The existence of working examples; and

(H) The quantity of experimentation needed to make or use the invention based on the content of the disclosure.

In re Wands, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). The examiner's analysis must consider all the evidence related to each of these factors, and any conclusion of nonenablement must be based on the evidence as a whole. 858 F.2d at 737, 740, 8 USPQ2d at

1404, 1407. Detailed procedures for making and using the invention may not be necessary if the description of the invention itself is sufficient to permit those skilled in the art to make and use the invention (M.P.E.P. §2164).

The claimed method is directed to *in vivo* suppression in a mammal of multiplicity in the stomach, colon and in both the stomach and colon of cancer cells which comprises: providing an effective amount of a composition which consists essentially of malvidin as an active ingredient to the mammal so as to suppress the multiplicity of the cells. The claim breadth is limited to multiplicity in the stomach and/or colon. The composition provided to the mammal is narrowly limited to a composition which consists essentially of malvidin. As noted in MPEP §2164.05(a), whether the specification would have been enabling as of the filing date involves consideration of the nature of the invention, the state of the prior art, and the level of skill in the art. The initial inquiry is into the nature of the invention, *i.e.*, the subject matter to which the claimed invention pertains. The subject

matter relates to suppression in a mammal of multiplicity of cancer cells in the stomach and/or colon.

The nature of the invention becomes the backdrop to determine the state of the art and the level of skill possessed by one skilled in the art. The relative skill of those in the art refers to the skill of those in the art in relation to the subject matter to which the claimed invention pertains at the time the application was filed (MPEP §2164.05(b)). The level of one of ordinary skill relating the suppression of a multiplicity of cancer cells in the stomach and/or colon was high at the time the application was filed. This is clear after considering the abstract of Barranco *et al.*, *Invest. New Drugs* 1: 117-127, 1983, and Barranco *et al.*, *Cancer Res.* 43: 1703-1709, 1983, where it is stated that their clones of a human adenocarcinoma of the stomach can provide valuable insight into the design of clinical protocols for treatment of gastric carcinoma in humans. (Declaration, filed June 29, 2006: Abstracts of Barranco *et al.* in Exhibit A). Thus, it is clear that there is a high

level of skill in this field, since specific cellular models have been established that relate to specific cancers.

According to MPEP 2164.03, the amount of guidance or direction needed to enable the invention is inversely related to the amount of knowledge in the state of the art as well as the predictability in the art. *In re Fisher*, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970). The "predictability or lack thereof" in the art refers to the ability of one skilled in the art to extrapolate the disclosed or known results to the claimed invention. What is known in the art provides evidence as to the question of predictability. The scope of the required enablement varies inversely with the degree of predictability involved, but even in unpredictable arts, a disclosure of every operable species is not required.

Enclosed is a Supplemental Declaration under 37 C.F.R. §1.132, filed October 6, 2006, showing the predictability in the art relating to *in vivo* use of anthocyanins and cyanidin in suppressing multiplicity

of human cancer cells of the stomach and/or colon. Kang *et al. Cancer Letters* 194 (2003) 13-19 (Supplemental Declaration: Exhibit A) shows that there is a direct correlation between *in vitro* and oral *in vivo* use in suppressing multiplicity of human cancer cells of the stomach or colon with anthocyanins and cyanidin. (Supplemental Declaration: Paragraph 1). Malvidin is a related compound to cyanidin (Supplemental Declaration: Paragraph 1 in reference to Figures 1 and 2 of the specification). Therefore, there is predictability in the art of *in vivo* suppression of multiplicity of cancer cells of the stomach and/or colon, where *in vitro* tests have been performed in that show the activity of a cancer treatment against stomach (AGS) or colon (HCT-116) cancer cell lines.

Malvidin and pelargonidin were in particular found to be excellent inhibitors of stomach and colon cancer cell lines *in vitro*. (Specification: paragraph 0043 on page 15; Figure 7). As illustrated in Figure 7, the percentage of cell viability of the stomach (AGS) and colon (HCT-116) cancer cell lines dropped below

about 40% when 200 ppm malvidin was added. Enclosed is a Declaration under 37 C.F.R. §1.132, filed June 29, 2006, that shows that the cell lines HCT-116 and AGS are recognized as correlating to the specific conditions of human colon cancer and human stomach cancer, respectively.

The colon cell line designated HCT-116 and the stomach cell line designated AGS were purchased from the American Type Culture Collection (Rockville, Md.). HCT-116 is a human cell line derived from a colorectal carcinoma of the colon, and AGS is a human cell line derived from a gastric adenocarcinoma of the stomach. (Declaration, filed June 29, 2006: Paragraphs 4 and 6 referring to ATCC Product Descriptions of Exhibit A). HCT-116 is an *in-vitro* model that is recognized as correlating to the specific condition of colon cancer as evidenced by Gieseg *et al.* "The influence of tumor size and environment on gene expression in commonly used human tumor lines", *BMC Cancer.* 2004 Jul 15;4:35. (Declaration, filed June 29, 2006: Paragraph 7). AGS is an *in-vitro* model that is recognized in the art as correlating to the specific

condition of stomach cancer as evidenced by Barranco *et al.* "Heterogeneous responses of an *in vitro* model of human stomach cancer to anticancer drugs", *Invest. New Drugs* 1: 117-127, 1983, and Barranco *et al.* "Establishment and characterization of an *in vitro* model system for human adenocarcinoma of the stomach", *Cancer Res.* 43: 1703-1709, 1983. (Declaration, filed June 29, 2006: Paragraph 8).

According to MPEP § 2164.02, the issue of "correlation" is related to the issue of the presence or absence of working examples. "Correlation" refers to the relationship between *in vitro* or *in vivo* animal model assays and a disclosed or a claimed method of use. An *in vitro* or *in vivo* animal model example in the specification, in effect, constitutes a "working example" if that example "correlates" with a disclosed or claimed method invention. If the art is such that a particular model is recognized as correlating to a specific condition, then it should be accepted as correlating unless the examiner has evidence that the model does not correlate. Even with such evidence, the examiner must weigh the evidence for and against

correlation and decide whether one skilled in the art would accept the model as reasonably correlating to the condition. *In re Brana*, 51 F.3d 1560, 1566, 34 USPQ2d 1436, 1441 (Fed. Cir. 1995) (reversing the PTO decision based on finding that *in vitro* data did not support *in vivo* applications).

Since the initial burden is on the examiner to give reasons for the lack of enablement, the examiner must also give reasons for a conclusion of lack of correlation for an *in vitro* or *in vivo* animal model example. A rigorous or an invariable exact correlation is not required. *Cross v. Iizuka*, 753 F.2d 1040, 1050, 224 USPQ 739, 747 (Fed. Cir. 1985). Given that the particular cell lines taught in the Examples are recognized as models correlating to the specific conditions of the claimed methods, it is believed that the specification provides enablement for the claimed methods for *in vivo* inhibition in a mammal.

M.P.E.P. § 2164.02 also states that the lack of working examples or lack of evidence that the claimed invention works as described should never be

the sole reason for rejecting the claimed invention on the grounds of lack of enablement. An applicant need not have actually reduced the invention to practice prior to filing. Compliance with the enablement requirement of 35 U.S.C. §112, first paragraph, does not turn on whether an example is disclosed. The specification need not contain an example if the invention is otherwise disclosed in such manner that one skilled in the art will be able to practice it without an undue amount of experimentation. *In re Borkowski*, 422 F.2d 904, 908, 164 USPQ 642, 645 (CCPA 1970).

The quantity of experimentation needed to be performed by one skilled in the art is only one factor involved in determining whether "undue experimentation" is required to make and use the invention. "[A]n extended period of experimentation may not be undue if the skilled artisan is given sufficient direction or guidance." *In re Colianni*, 561 F.2d 220, 224, 195 USPQ 150, 153 (CCPA 1977). "The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely

routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed." *In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988) (citing *In re Angstadt*, 537 F.2d 489, 502-04, 190 USPQ 214, 217-19 (CCPA 1976)).

For example, as noted in M.P.E.P. §2164.01(c), it is not necessary to specify the dosage or method of use if it is known to one skilled in the art that such information could be obtained without undue experimentation. If one skilled in the art, based on knowledge of compounds having similar physiological or biological activity, would be able to discern an appropriate dosage or method of use without undue experimentation, this would be sufficient to satisfy 35 U.S.C. 112, first paragraph. The fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation. *In re Certain Limited-Charge Cell Culture Microcarriers*, 221 USPQ 1165, 1174 (Int'l Trade Comm"n 1983), *aff'd. sub nom., Massachusetts Institute of Technology v. A.B. Fortia*, 774 F.2d 1104, 227 USPQ

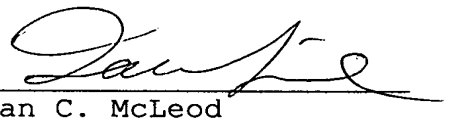
428 (Fed. Cir. 1985). See also *In re Wands*, 858 F.2d at 737, 8 USPQ2d at 1404. The test of enablement is not whether any experimentation is necessary, but whether if experimentation is necessary, it is undue. *In re Angstadt*, 537 F.2d 498, 504, 190 USPQ 214, 219 (CCPA 1976).

The determination that "undue experimentation" would have been needed to make and use the claimed invention is not a single, simple factual determination. Rather, it is a conclusion reached by weighing all the above noted factual considerations. *In re Wands*, 858 F.2d at 737, 8 USPQ2d at 1404. Considering all of the above noted factual considerations, undue experimentation would not have been needed to make and use the claimed invention. Therefore, the claimed method for *in vivo* suppression in a mammal of multiplicity in the stomach, colon and in both the stomach and colon of cancer cells is enabled by the specification. Reversal of the rejection is requested.

(B.) Conclusion

As shown above, the claimed subject matter was described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. Therefore, Claims 1 and 5-7 are each patentable. Reversal of the Final Rejection is requested.

Respectfully,



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CLAIMS APPENDIX

1. A method for *in vivo* suppression in a mammal of multiplicity in the stomach, colon and in both the stomach and colon of cancer cells which comprises:

providing an effective amount of a composition which consists essentially of malvidin as an active ingredient to the mammal so as to suppress the multiplicity of the cells.

5. The method of Claim 1 wherein the cells are in a mammal and the malvidin is fed orally to the mammal.

6. The method of Claim 1 wherein the composition is in a pharmaceutical carrier.

7. The method of Claim 1 wherein the stomach cell is AGS and the colon cell is HCT 116 both as maintained by the American Type Culture Collection.

EVIDENCE APPENDIX

1. Enclosed is a Declaration under 37 C.F.R. §1.132, filed June 29, 2006, that shows that the cell lines HCT-116 and AGS are recognized as correlating to the specific conditions of human colon cancer and human stomach cancer, respectively. The Declaration was considered by Examiner Flood as noted on page 2 of the Office Action mailed July 25, 2006. Also attached are ATCC product descriptions, an article by Gieseg *et al.*, and abstracts by Barranco *et al.* filed as Exhibit A of the Declaration.

2. Enclosed is a Supplemental Declaration under 37 C.F.R. §1.132, filed October 6, 2006, that supports the conclusion that *in vivo* results with malvidin are predictive of *in vivo* activity suppressing multiplicity of cancer cells. The Declaration was considered by Examiner Flood as noted on page 2 of the Final Office Action mailed January 10, 2007. Also attached is an article by Kang *et al.* *Cancer Letters* 194 (2003) 13-19, filed as Exhibit A of the Supplemental Declaration.

RELATED PROCEEDINGS APPENDIX

(None.)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appln. No. : 10/725,214 Confirmation No. 4443

Applicants : Muraleedharan G. Nair, Yanjun Zhang and Shaiju K. Vareed

Filed : December 1, 2003

Title : METHOD FOR INHIBITING CANCER CELLS

TC/A.U. : 1655

Examiner : Michele C. Flood

Docket No. : MSU 4.1-672

Customer No. : 21036

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DECLARATION UNDER 37 C.F.R. § 1.132

Dear Sir:

Muraleedharan G. Nair states as follows:

(1.) That he is an inventor of the invention in the above entitled application.

(2.) That he is a Professor of Horticulture & Food Safety & Toxicology at Michigan State University, East Lansing, Michigan 48824.

(3.) That the colon cell line designated "HCT-116" was purchased from the American Type Culture Collection (Rockville, Md.).

(4.) That "HCT-116" is a human cell line derived from a colorectal carcinoma of the colon as evidenced by the attached product description of the American Type Culture Collection (Rockville, Md.).

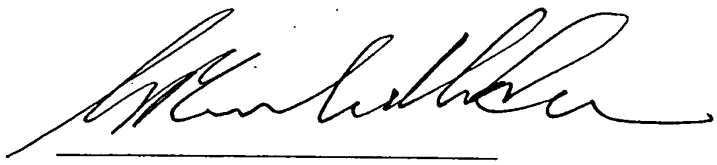
(5.) That the stomach cell line designated "AGS" was purchased from the American Type Culture Collection (Rockville, Md.).

(6.) That "AGS" is a human cell line derived from a gastric adenocarcinoma of the stomach as evidenced by the attached product description of the American Type Culture Collection (Rockville, Md.).

(7.) That "HCT-116" is an *in-vitro* model that is recognized as correlating to the specific condition of colon cancer as evidenced by the enclosed publication, Gieseg *et al.* "The influence of tumor size and environment on gene expression in commonly used human tumor lines", *BMC Cancer*. 2004 Jul 15;4:35.

(8.) That "AGS" is an *in-vitro* model that is recognized in the art as correlating to the specific condition of stomach cancer as evidenced by the enclosed abstracts, Barranco *et al.* "Heterogeneous responses of an *in vitro* model of human stomach cancer to anticancer drugs", *Invest. New Drugs* 1: 117-127, 1983, and Barranco *et al.* "Establishment and characterization of an *in vitro* model system for human adenocarcinoma of the stomach", *Cancer Res.* 43: 1703-1709, 1983.

(9.) That the undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.



Muraleedharan G. Nair

Date: 6/23/06

EXHIBIT A

Product Description

Before submitting an order you will be asked to read and accept the terms and conditions of ATCC's Material Transfer Agreement or, in certain cases, an MTA specified by the depositing institution.

Customers in Europe, Australia, Hong Kong, India, Japan, Korea, New Zealand, Singapore and Taiwan, R.O.C. must contact a local distributor for pricing information and to place an order for ATCC cultures and products.

Cell Biology

ATCC® Number:	CCL-247™ Order this item	**Price:**	$203.00
Designations:	HCT 116	**Depositors:**	MG Brattain
Biosafety Level:	1	**Shipped:**	frozen
Medium & Serum:	See Propagation	**Growth Properties:**	adherent
Organism:	*Homo sapiens* (human)	**Morphology:**	epithelial PHOTO
Source:	**Organ:** colon **Disease:** colorectal carcinoma		
Cellular Products:	carcinoembryonic antigen (CEA) 1 ng per 10 exp6 cells per 10 days; keratin		
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.		

Related Cell Culture Products

Applications:	transfection host (technology from amaxa)
Tumorigenic:	Yes, in nude mice [23040]
Reverse Transcript:	negative
DNA Profile (STR):	Amelogenin: X,Y CSF1PO: 7,10 D13S317: 10,12 D16S539: 11,13 D5S818: 10,11 D7S820: 11,12 THO1: 8,9 TPOX: 8,9 vWA: 17,22
Cytogenetic Analysis:	The stemline chromosome number is near diploid with the modal number at 45 (62%) and polyploids occurring at 6.8%. The markers 10q+ and t(?8p;18q) are present in all metaphases and t(9q;?16p-), in 80% of the cells karyotyped. N16 is monosomic in the presence of, but disomic in the absence of t(9q;?16p-). N10 and N18 are monosomic and other chromosomes from those mentioned above are disomic. Q-band observations revealed the presence of the Y chromosome, but not in all cells (50% of cells lacked the Y in G-band karyotypes).
Isoenzymes:	AK-1, 1; ES-D, 1-2; G6PD, B; GLO-I, 1; PGM1, 1; PGM3, 1
Age:	adult

Gender:	male
Comments:	The cells are positive for keratin by immunoperoxidase staining. HCT 116 cells are positive for transforming growth factor beta 1 (TGF beta 1) and beta 2 (TGF beta 2) expression. This line has a mutation in codon 13 of the ras protooncogene, and can be used as a positive control for PCR assays of mutation in this codon.
Propagation:	**ATCC complete growth medium:** McCoy's 5a medium (modified) with 1.5 mM L-glutamine adjusted to contain 2.2 g/L sodium bicarbonate, 90%; fetal bovine serum, 10% **Temperature:** 37.0C **Atmosphere:** air, 95%; carbon dioxide (CO2), 5% **Growth Conditions:** Growth and plating efficiency are enhanced by using a feeder layer of murine fibroblasts. [26071]
Subculturing:	**Protocol:** 1. Remove and discard culture medium. 2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin- 0.53 mM EDTA solution to remove all traces of serum which contains trypsin inhibitor. 3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes). Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal. 4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting. 5. Add appropriate aliquots of the cell suspension to new culture vessels. 6. Incubate cultures at 37°C. **Subcultivation ratio:** A subcultivation ratio of 1:3 to 1:8 is recommended **Medium renewal:** Every 2 to 3 days
Preservation:	**Freeze medium:** Complete growth medium supplemented with 5% (v/v) DMSO **Storage temperature:** liquid nitrogen vapor phase
Related Products:	Recommended medium (without the additional supplements or serum described under ATCC Medium): ATCC 30-2007 recommended serum: ATCC 30-2020 feeder layer cells: ATCC 56-X
References:	22794: Schroy PC , et al. Detection of p21ras mutations in colorectal adenomas and carcinomas by enzyme-linked immunosorbent assay. Cancer 76: 201-209, 1995. PubMed: 8625092 23040: Brattain MG , et al. Heterogeneity of malignant cells from a human colonic carcinoma. Cancer Res. 41: 1751-1756, 1981. PubMed: 7214343 23125: Sun L , et al. Autocrine transforming growth factor-beta 1 and beta 2 expression is increased by cell crowding and quiescence in colon carcinoma cells. Exp. Cell Res. 214: 215-224, 1994. PubMed: 8082724 25093: Santoro IM , Groden J . Alternative splicing of the APC gene and its association with terminal differentiation. Cancer Res. 57: 488-494, 1997. PubMed: 9012479 26071: Brattain MG , et al. Enhancement of growth of human colon tumor cell lines by feeder layers of murine fibroblasts. J. Natl. Cancer Inst. 69: 767-771, 1982. PubMed: 6956756 32266: Bender CM , et al. Inhibition of DNA methylation by 5-Aza-2'-deoxycytidine suppresses the growth of human tumor cell lines. Cancer Res. 58: 95-101, 1998. PubMed: 9426064 32288: Landers JE , et al. Translational enhancement of mdm2 oncogene expression in human tumor cells containing a stabilized wild-type p53 protein. Cancer Res. 57: 3562-3568, 1997. PubMed: 9270029 32794: Kutchera W , et al. Protaglandin H synthase 2 is expressed abnormally in human colon cancer: evidence for a transcriptional effect. Proc. Natl. Acad. Sci. USA 93: 4816-4820, 1996. PubMed: 8643486 32910: Wang R , et al. Cellular adherence elicits ligand-independent activation of the Met cell-surface receptor. Proc. Natl. Acad. Sci. USA 93: 8425-8430, 1996. PubMed: 8710887

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Product Description

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Cell Biology

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Designations:	AGS	**Depositors:**	SC Barranco
Biosafety Level:	1	**Shipped:**	frozen
Medium & Serum:	See Propagation	**Growth Properties:**	adherent
Organism:	*Homo sapiens* (human)	**Morphology:**	epithelial
Source:	**Organ:** stomach **Disease:** gastric adenocarcinoma		
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.		

Related Cell Culture Products

Isolation:	**Isolation date:** 1979
Applications:	transfection host (technology from amaxa)
Tumorigenic:	Yes, in athymic BALB/c mice
Cytogenetic Analysis:	This is a hyperdiploid human cell line. The modal chromosome number was 49, occurring in 60% of cells. The rate of polyploidy was 3.6%. Single copy each for der(8)t(1;8) (q12;p23), der(19)t(19;?) (q13.6;?), minute chromosome M3, and C-group-like M12 was seen in all cells. The origins of both M3 and M12 defied identification presently. The t(13q14q) occurred in some. Generally there were three copies for N20, and single copy for X, N8 and N18. Occasionally there were three copies for N14.
Age:	54 years
Gender:	female
Ethnicity:	Caucasian
Comments:	The AGS cell line was derived from fragments of a tumor resected from a patient who had received no prior therapy. The cells have a plating efficiency of 34% in the medium below. The line was cured at the ATCC of a prior mycoplasma infection.
Propagation:	**ATCC complete growth medium:** Ham's F12K medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 90%; fetal bovine serum, 10% **Temperature:** 37.0C
Subculturing:	**Protocol:** 1. Remove and discard culture medium. 2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin- 0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor. 3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope

	until cell layer dispersed (usually within 5 to 15 minutes). Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal. 4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting. 5. Add appropriate aliquots of the cell suspension to new culture vessels. 6. Incubate cultures at 37°C. **Subcultivation ratio:** A subcultivation ratio of 1:3 to 1:8 is recommended **Medium renewal:** Every 2 to 3 days
Preservation:	**Freeze medium:** Complete growth medium, 95%; DMSO, 5% **Storage temperature:** liquid nitrogen vapor phase
Doubling Time:	20 hrs
Related Products:	Recommended medium (without the additional supplements or serum described under ATCC Medium): ATCC 30-2004 recommended serum: ATCC 30-2020
References:	22748: Barranco SC , et al. Heterogeneous responses of an in vitro model of human stomach cancer to anticancer drugs. Invest. New Drugs 1: 117-127, 1983. PubMed: 6678861 23044: Barranco SC , Townsend CM Jr. Establishment and characterization of an in vitro model system for human adenocarcinoma of the stomach. Cancer Res. 43: 1703-1709, 1983. PubMed: 6831414 32252: Rieder G , et al. Role of adherence in Interleukin-8 induction in Helicobacter pylori-associated gastritis. Infect. Immun. 65: 3622-3630, 1997. PubMed: 9284128 32464: Segal ED , et al. Helicobacter pylori attachment to gastric cells induces cytoskeletal rearrangements and tyrosine phosphorylation of host cell proteins. Proc. Natl. Acad. Sci, USA 93: 1259-1264, 1996. PubMed: 8577751

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The influence of tumor size and environment on gene expression in commonly used human tumor lines

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Abstract

Background: The expression profiles of solid tumor models in rodents have been only minimally studied despite their extensive use to develop anticancer agents. We have applied RNA expression profiling using Affymetrix U95A GeneChips to address fundamental biological questions about human tumor lines.

Methods: To determine whether gene expression changed significantly as a tumor increased in size, we analyzed samples from two human colon carcinoma lines (Colo205 and HCT-116) at three different sizes (200 mg, 500 mg and 1000 mg). To investigate whether gene expression was influenced by the strain of mouse, tumor samples isolated from C.B-17 SCID and Nu/Nu mice were also compared. Finally, the gene expression differences between tissue culture and *in vivo* samples were investigated by comparing profiles from lines grown in both environments.

Results: Multidimensional scaling and analysis of variance demonstrated that the tumor lines were dramatically different from each other and that gene expression remained constant as the tumors increased in size. Statistical analysis revealed that 63 genes were differentially expressed due to the strain of mouse the tumor was grown in but the function of the encoded proteins did not link to any distinct biological pathways. Hierarchical clustering of tissue culture and xenograft samples demonstrated that for each individual tumor line, the *in vivo* and *in vitro* profiles were more similar to each other than any other profile. We identified 36 genes with a pattern of high expression in xenograft samples that encoded proteins involved in extracellular matrix, cell surface receptors and transcription factors. An additional 17 genes were identified with a pattern of high expression in tissue culture samples and encoded proteins involved in cell division, cell cycle and RNA production.

Conclusions: The environment a tumor line is grown in can have a significant effect on gene expression but tumor size has little or no effect for subcutaneously grown solid tumors. Furthermore, an individual tumor line has an RNA expression pattern that clearly defines it from other lines even when grown in different environments. This could be used as a quality control tool for preclinical oncology studies.

Background

Preclinical animal models of human tumors represent a major tool for the selection and development of effective anticancer agents. There is a considerable number of well characterized human cancer cell lines, many of which can be grown as solid tumors (either subcutaneously or orthotopicaly) in immunodeficient mice. The ease of use and low cost of these models make them desirable for screening *in vivo* activity of anticancer compounds as compared to induced or transgenic rodent tumor models. Rodent models also allow pharmacodynamic and pharmacokinetic parameters to be directly measured and related to antitumor efficacy. Commonly used human cancer cell lines, such as the panel of 60 lines (NCI60) used by the Developmental Therapeutics Program at the National Cancer Institute, have been extensively studied at the molecular level *in vitro* and to a lesser extent *in vivo*. Recent advances in genomic technology allow molecular characterization of these models to an extent never before possible using RNA expression profiling, comparative genomic hybridization, proteomic and metabonomic profiling. Ross *et al.* [1] looked at the *in vitro* gene expression in the NCI60 panel. The gene expression pattern for many lines indicated a relationship with the tumor tissue of origin and also correlated with doubling time and drug metabolism. Virtanen *et al.* [2] have analyzed the expression patterns of 85 lung tumor samples from both clinical samples and established tumor lines. The fresh tumors clustered according to pathological subtype with many of the cell lines also clustering within the same groups. Pedersen *et al.* [3] performed a similar study with human small cell lung cancer cell lines and compared them to ressected tissue samples. Dan *et al.* [4] recently performed RNA expression profiling on 39 human cancer cell lines and related the gene expression patterns to chemosensitivity of 55 anticancer agents. Zembutsu *et al.* [5] performed a similar study but profiled 85 human cancer xenografts.

What was apparent in all these studies was that each tumor model was distinctly different and could be distinguished from related models using routine data analysis methods. Furthermore, it appears that it is possible to identify models that are no longer true to their origins. Ross *et al.* [1] identified MDA-MB-435 as a possible melanoma derived line, which is at odds with its supposed origins of a metastatic breast carcinoma [6]. However, such identification also raises the possibility that the sample that Ross *et al.* obtained was not the true "MDA-MB-435." Clearly there is the opportunity to use profiling technology as an advanced quality control method that could not only identify mislabeled tumor lines but possibly genetic drift.

While these reports have tremendous value they do not address some basic questions about human tumor models that could impact the design of *in vivo* drug development studies. For example, most, if not all, xenografts demonstrate Gompertzian growth kinetics with continuously increasing doubling times as they grow larger [7]. Despite this obvious change in biology, it is unknown whether xenografts alter their expression as they increase in size or whether expression differences exist for the same tumor line grown both as a solid tumor in a mouse or in tissue culture. To investigate these questions we grew human colon tumors (HCT-116 and Colo205) in Nu/Nu mice and harvested tumors at three sizes (200 mg, 500 mg, 1000 mg). Isolated RNA was analyzed on Affymetrix GeneChips. The effect of mouse strain on tumor gene expression was also investigated by comparing tumors at 500 mg grown in Nu/Nu and C.B-17 SCID mouse strains. Lastly we profiled additional tumor models to investigate the changes in gene expression that occur when a tumor line is grown *in vivo* or *in vitro*.

Methods

Xenograft and tissue culture methods

Cell lines were grown in DMEM/F12 supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, California). They were passaged in 75-cm^2 tissue culture flasks in an atmosphere of 5% CO_2 in air and were subcultured weekly, using 0.05% trypsin EDTA (Invitrogen, Carlsbad, California). RNA from tissue culture samples was harvested at mid-log phase. Human tumor xenografts were grown in either Nu/Nu mice or C.B-17 SCID female mice obtained from Charles River Laboratories (Wilmington, Massachusetts) between four and five weeks of age. Mice were housed five to a cage in animal rooms maintained at between 21–25°C with a 12 h alternating dark/light cycle. All animal studies were conducted under Veterinary Use Protocols approved by the Institutional Animal Care and Use Committee. Tumors were maintained by serial passage of 30 mg tumor fragments between animals, implanted subcutaneously into the right axillary region using a trocar needle aseptically. Tumors were passed when the primary had reached between 500 and 1000 mg and were never passed more than ten times. Tumor growth was followed by caliper measurements of perpendicular measures of the tumor. The weight in mg was estimated by the formula: tumor weight = $a(b^2)/2$, where a and b are the tumor length and width respectively in mm. Tumor tissue was harvested immediately following animal sacrifice by excising the tumor and powdering it in a liquid nitrogen cooled crucible and pestle. Tumor powder was stored at -80°C until RNA isolation.

Affymetrix GeneChip

RNA was extracted using TRIZOL® reagent (Invitrogen, Carlsbad, California) according to the manufacturer's protocol. RNA integrity was monitored using denaturing agarose gel electrophoresis in 1X MOPS. Biotinylated target

RNA was prepared from 15 μg of total RNA using the Affymetrix protocol. Briefly, double-stranded cDNA was prepared from the RNA template using a modified oligo-dT primer containing a 5' T7 RNA polymerase promoter sequence and the Superscript Choice System for cDNA Synthesis (Invitrogen, Carlsbad, California). Following phenol-chloroform extraction and ethanol precipitation, one-half of the cDNA reaction (0.5 – 1.0 μg) was used as the template in an *in vitro* transcription reaction containing T7 RNA polymerase, a mixture of unlabeled ATP, CTP, GTP, and UTP, and biotin-11-CTP and biotin-16-UTP (BioArray High Yield Kit, ENZO, Farmingdale, New York). The resulting biotinylated-cRNA "target" was purified on an affinity resin (RNeasy, Qiagen, Valencia, California) and quantified using the convention that 1 O.D. 260 nm corresponds to 40 μg/mL of RNA. Typical yields ranged from 50 – 100 μg with transcript sizes between 3.0 to 0.25 kilonucleotides as determined by denaturing gel electrophoresis. Fifteen micrograms of biotinylated cRNA was randomly fragmented to an average size of 50 nucleotides by incubating at 94°C for 35 minutes in 40 mM TRIS-acetate, pH 8.1, 100 mM potassium acetate, and 30 mM magnesium acetate. The fragmented cRNA was hybridized in a solution containing 100 mM MES, 1 M [Na^+], 20 mM EDTA, 0.01% TWEEN 20, 50 pM of Control Oligonucleotide B2, 0.1 mg/mL of sonicated herring sperm DNA, and 0.5 mg/mL BSA for 16 hours at 45°C on either the Human U95A or the Mouse U74A Affymetrix GeneChips® (Affymetrix, Santa Clara, California). Each hybridization included a mixture of four bacterial biotinylated-RNA transcripts (BioB, BioC, BioD, and cre) spiked at 1.5, 5, 25, and 100 pM, respectively. The hybridization reactions were processed and scanned according to the standard Affymetrix protocols.

Data analysis

All arrays were global scaled to a target intensity value of 600 using the standard Affymetrix protocol. Calculation of the scaling factor, background, noise and percent present, was performed according to Affymetrix protocols using the Data Mining Tool (Affymetrix Santa Clara, California). All resulting data sets were filtered using the absolute call metric (present or absent) using Microsoft Access (Microsoft Corporation, Redmond, Washington). Genes selected had expression levels classified as present at least once in the samples selected for the particular analysis.

To determine the relationship between tumor samples harvested at different sizes the filtered RNA profiling data was analyzed with classic multidimensional scaling (MDS), implemented in R [8,9]. MDS is an unsupervised learning technique that attempts to preserve the relationship between points from high dimensional space at lower dimensional spaces. The program R is a free integrated suite of software facilities for data manipulation, calculation and graphical display http://www.r-project.org.

Analysis of variance (ANOVA) was used to test the effects of tumor size and tumor line, using the following model: $Expression\ of\ gene_i \sim tumor.size + tumor.line + tumor.line*tumor.size$. To test the effect due to mouse background, we used the following model: $Expression\ of\ gene_i \sim mouse\ background$. RNA profiling data filtered on the absolute call metric was used for this analysis and ANOVA was implemented in R. ANOVA is a statistical linear modeling procedure that partitions the total variance into parts corresponding to various sources in the model [10,11]. It has been used previously in microarray data analysis [12-15].

To rigorously select genes with expression differences between samples, ANOVA p-values were adjusted using multiple comparison procedures. Multiple comparison procedures are tools to adjust p-values that might be inflated as a result of performing multiple hypothesis tests. The Benjamini and Hochberg procedure controls the false discovery rate, which is the expected fraction of false discoveries in all rejected hypothesis [16]. This procedure is less stringent than methods controlling the family wise error rate (e.g. the Bonferroni correction); hence it is more powerful.

Hierarchical clustering was performed in GeneSpring 5.0 (Silicon Genetics, Redwood City, California). The distinction calculation from Spotfire DecisionSite 6.2 (Spotfire Inc. Somerville, Massachusetts) was used to select genes differentially expressed in xenograft samples or tissue culture samples. All data from the tissue culture samples that had an *in vivo* pair (8 samples) were selected into one group and all data from the xenograft samples (8 samples) were selected into a second group. Genes were prefiltered using the absolute call metric by selecting genes that were present at least once in the selected samples. A distinction value score and p-value was calculated for each gene. The score (≥ 1) and p-value (≤ 0.001) was then used to select genes that were differentially expressed between xenograft samples and tissue culture samples. To functionally classify gene lists, web resources such as NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/) were searched and the data compiled. Further searching for gene associations in PubMed (NCBI) was also performed.

Results

Variation in tumor xenograft gene expression due to size

We focused on two human colon carcinoma xenografts (HCT-116 and Colo205) to investigate the effects of tumor size and mouse strain on gene expression. Samples were harvested in quadruplicate at three different tumor sizes (200 mg, 500 mg and 1000 mg) for both tumor

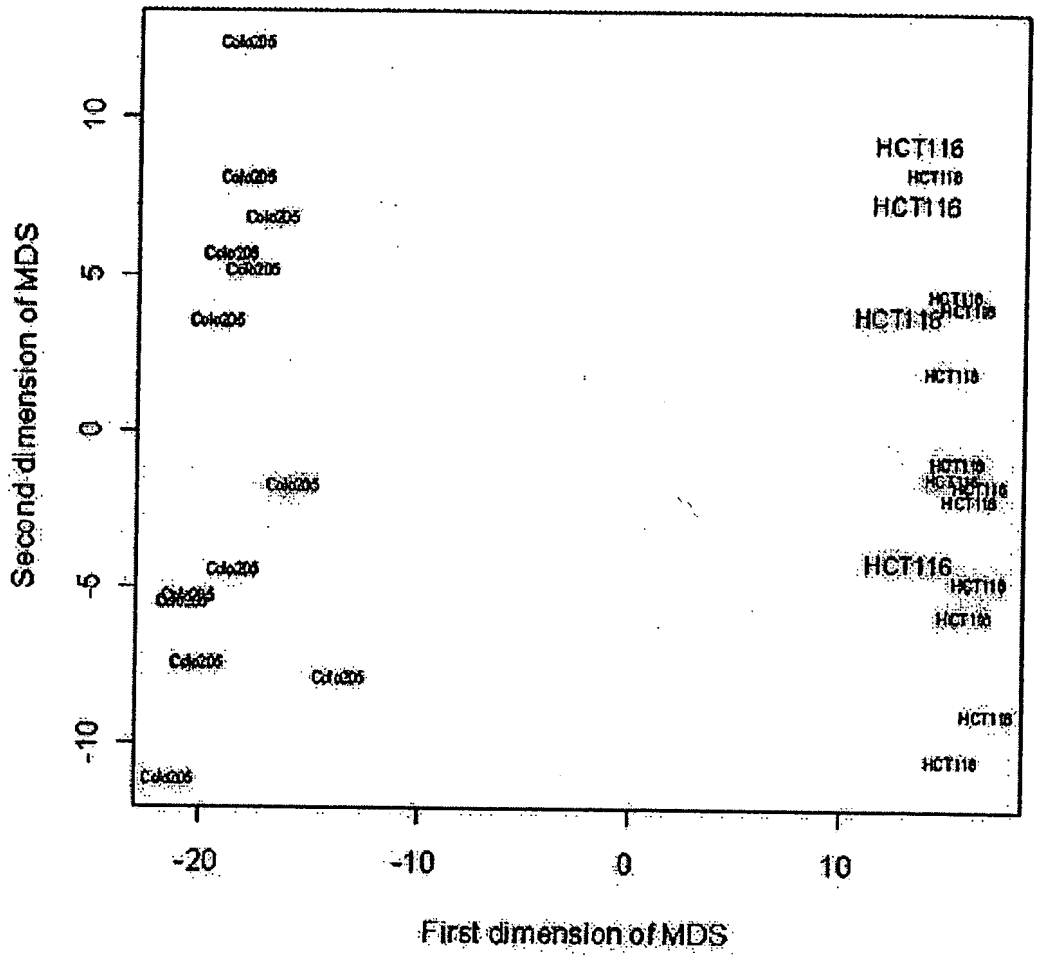

Figure 1

Multidimensional scaling plot of Colo205 and HCT-116 samples. Multidimensional scaling plot showing the relatedness of individual samples from Colo205 and HCT-116 to each other in 2D space. The color indicates the size of the tumor sample when harvested: red, 200 mg; blue, 500 mg; black, 1000 mg. The size of text indicates the mouse strain the tumor was grown in: small, Nu/Nu; large, C.B-17 SCID. Samples that are more related to each other are closer together. For Colo205 five samples were analyzed.

models grown in Nu/Nu mice (except for the 500 mg sample of Colo205 where five samples were harvested). These sizes were selected as they represent the range at which sensitivity to anticancer agents are traditionally tested and because most models approximate log-linear growth at these sizes. RNA expression profiling data was obtained from Affymetrix U95A GeneChips containing approximately 12600 genes. Genes present (above background) once or more across all samples were selected for further analysis (approximately 7600 genes).

Initial analysis of the expression data with multi-dimensional scaling (MDS) showed that samples from the same tumor line clustered together and that there was clear separation between samples from HCT-116 and Colo205 (Figure 1). Compared to the profound effect due to tumor

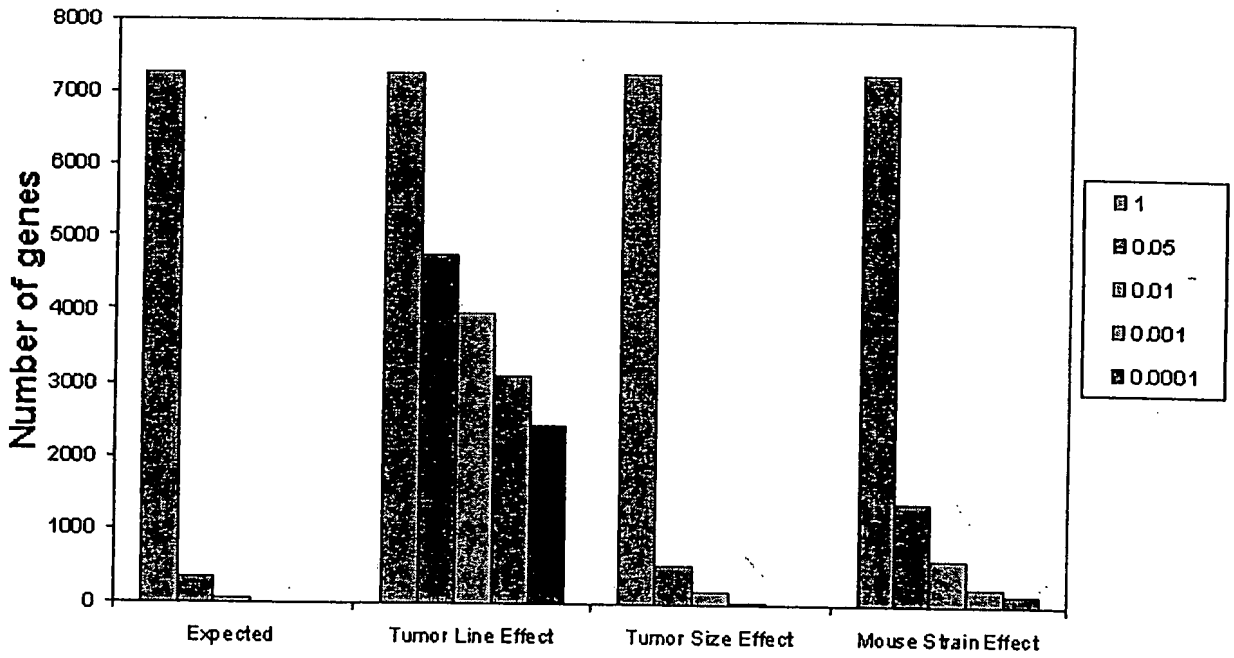

Figure 2

Expected verses observed number of significantly changed genes. The graph shows the number of genes (y-axis) that fall into specific categories (x-axis) based on the ANOVA calculated p-values. The categories are as follows: *Predicted*, number of genes expected by chance alone; *Tumor line effect*, number of genes that fall within the specified ranges due to differences in the tumor line; *Tumor size effect*, number of genes that fall within the specified ranges due to changes in the tumor size; *Mouse strain effect*, number of genes that fall within the specified range due to changes in the mouse strain the tumors were grown in.

line, there was no clear separation among samples of different sizes in the MDS plot, suggesting that there was little alteration in gene expression due to differences in tumor size (tumor size effect).

The result from MDS was further confirmed by analysis of variance (ANOVA). Using ANOVA we modeled the effects of tumor line and tumor size on gene expression. Since in the ANOVA we conducted approximately 7000 statistical tests (on the selected genes), with a p-value cutoff of 0.01, we would expect approximately 70 genes (1% of 7000) scored as significantly changed due to chance alone. Indeed, the observed number of significantly changed genes ($p \leq 0.01$) due to tumor size effect was 154, approximately twice what was predicted by chance alone (Figure 2). In contrast, the number of significantly changed genes due to tumor line effect (4731 genes p-value < 0.01) was far greater than chance alone, indicating the two tumor lines were extremely different as suggested by MDS. The distribution of p-values confirmed the profound effect of tumor line on gene expression with genes affected at all expression levels (Figure 3).

To rigorously identify genes that may suggest functionally significant changes as a tumor increases in size from 200 mg to 1000 mg the ANOVA p-values were adjusted using multiple comparison procedures. Following this analysis none of the previously identified 154 genes had p-values < 0.05. This indicated that there was no change in gene expression as a human tumor xenograft increased in size from 200 mg to 1000 mg. This was true for both the HCT-116 and the Colo205 tumor lines.

Variation in tumor xenograft gene expression due to mouse strain

Additional RNA samples were prepared from 500 mg HCT-116 tumors grown in C.B-17 SCID mice and hybridized to Affymetrix U95A GeneChips. The expression data were compared to data from the same line grown in Nu/Nu mice harvested at 500 mg. The MDS analysis showed there was no distinct separation or grouping for samples from the two different mouse strains (Figure 1). This suggests that mouse strain played only a small role in altering the expression profiles of HCT-116 tumor samples. However, ANOVA did reveal that considerably more genes

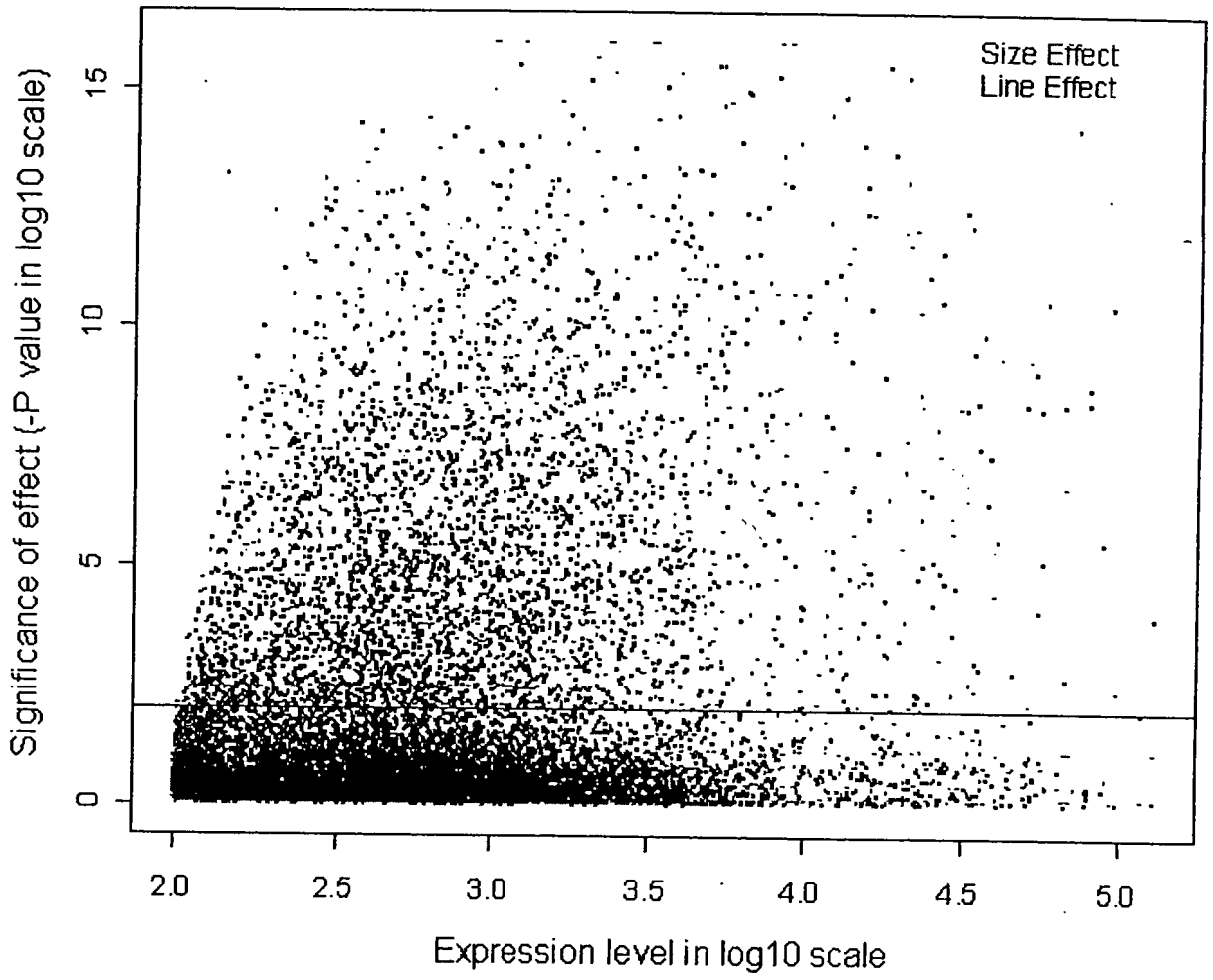

Figure 3

P-value distribution/volcano plot of line and size effect. Scatter plot with mean expression level (log 10) on the x-axis and – p-value (log 10) on y-axis. Each gene is plotted twice, once for the p-value resulting from the tumor-line effect ANOVA (black) and once for the p-value resulting from the tumor-size effect ANOVA (red). The blue line represents a p-value of 0.01. Genes with a lower p-value (more significant) have a higher – p-value log10. There are far more genes with significant p-values due to differences in the tumor lines than due to changes in tumor size.

were altered in their expression due to the mouse background (493 genes) compared to chance alone ($p < 0.01$). To identify genes that may suggest biologically significant differences due to the mouse background effect, the ANOVA p-values were adjusted using multiple comparison procedures. Using the Benjamini and Hockberg procedure [16] 63 genes were found to have a p-value < 0.05 with 32 genes increased in the C.B17-SCID strain and 31 increased in the Nu/Nu strain (Table 1 and 2). Functional classification of these genes using a gene ontology approach did not identify functions that could be linked to known biological pathways. Therefore a biological understanding of the changes in gene expression due to mouse strain remains elusive.

Table 1: Genes with high expression in HCT-116 tumors grown in C.B-17 SCID mice

Function	Affymetrix Probe Set ID	ANOVA p-value	BH p-value[1]	Log2 FC[2]	Gene Symbol	LocusLink ID	Sequence Probe Derived From	Gene Title
Translation/RNA binding/RNA splicing								
	34733_at	0.00029	0.046	0.5	SF3A1	10291	X85237	splicing factor 3a, subunit 1, 120 kDa
	34829_at	0.00020	0.046	0.4	DKC1	1736	U59151	dyskeratosis congenita 1, dyskerin
	35174_i_at	0.00020	0.046	0.4	EEF1A2	1917	X70940	eukaryotic translation elongation factor 1 alpha 2
	37462_i_at	0.00013	0.042	0.5	SF3A2	8175	L21990	splicing factor 3a, subunit 2, 66 kDa
	39047_at	0.00007	0.030	0.5	SART3	9733	AB020880	squamous cell carcinoma antigen recognised by T cells 3
	40490_at	0.00041	0.049	0.7	DDX21	9188	U41387	DEAD/H box polypeptide 21
Signal transduction								
	33887_at	0.00029	0.046	0.5	HGS	9146	D84064	hepatocyte growth factor-regulated tyrosine kinase substrate
	38019_at	0.00002	0.028	1.0	CSNK1E	1454	L37043	casein kinase 1, epsilon
	38779_r_at	0.00039	0.048	0.8	HDGF	3068	D16431	hepatoma-derived growth factor
	40864_at	0.00029	0.046	0.4	RAC1	5879	D25274	ras-related C3 botulinum toxin substrate
Small Molecule transport								
	32186_at	0.00023	0.046	0.7	SLC7A5	8140	M80244	solute carrier family 7, member 5
	36557_at	0.00003	0.028	0.9	CACNB1	782	M92303	calcium channel, voltage-dependent, beta 1 subunit
	38029_at	0.00031	0.046	0.4	SLC3A2	6520	J02939	solute carrier family, member 2
Protein Complex Assembly								
	31842_at	0.00044	0.050	0.5	BCS1L	617	AF038195	BCS1-like (yeast)
	32575_at	0.00019	0.046	0.4	NAP1L4	4676	U77456	nucleosome assembly protein 1-like 4
Protein transport and folding								
	36945_at	0.00036	0.047	0.5	C12orf8	10961	X94910	chromosome 12 open reading frame 8
	40756_at	0.00017	0.046	0.4	NPM3	10360	AF081280	nucleophosmin/nucleoplasmin, 3
Unknown or other functions								
	31670_s_at	0.00023	0.046	0.3	SRP72	6731	U81554	signal recognition particle 72 kDa
	34279_at	0.00005	0.030	2.0	FLJ20719	55672	AL050141	hypothetical protein FLJ20719
	36192_at	0.00003	0.028	0.7	KIAA0193	9805	D83777	KIAA0193 gene product
	36209_at	0.00003	0.028	0.8	BRD2	6046	S78771	bromodomain containing 2
	36210_g_at	0.00035	0.047	1.0	BRD2	6046	S78771	bromodomain containing 2
	36668_at	0.00035	0.047	0.4	DIA1	1727	M28713	diaphorase (NADH)
	37907_at	0.00006	0.030	0.4	F8A	8263	M34677	coagulation factor VIII-associated
	38885_at	0.00000	0.007	1.0	DNA2L	1763	D42046	DNA2 DNA replication helicase 2-like
	39436_at	0.00010	0.039	1.5	BNIP3L	665	AF079221	BCL2/adenovirus E1B 19 kDa interacting protein 3-like
	39758_f_at	0.00030	0.046	0.4	LAMP1	3916	J04182	lysosomal-associated membrane protein 1
	39832_at	0.00007	0.030	0.5	ARS2	51593	AL096723	arsenate resistance protein ARS2
	40589_at	0.00041	0.049	0.9	SNTB2	6645	U40572	syntrophin, beta 2
	40604_at	0.00012	0.040	1.1	DYRK2	8445	Y13493	dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2
	41062_at	0.00028	0.046	0.7	NSPC1	84759	AA037278	likely ortholog of mouse nervous system polycomb 1
	982_at	0.00011	0.039	0.4	MCM5	4174	X74795	MCM5 minichromosome maintenance deficient 5, cell division cycle 46

[1]p-value resulting from Benjamini and Hochberg procedure [2]log2 fold change calculated by dividing expression level from C.B-17 SCID sample by expression level of Nu/Nu sample

Comparison of lines grown in both tissue culture and solid tumor xenografts

To explore the relatedness of different tumor lines to each other and to investigate the gene expression differences between growth in tissue culture and growth as a subcutaneous solid tumor, RNA samples were prepared from the 13 human tumor lines grown in tissue culture to mid-log phase (Table 3). An additional eight RNA samples were obtained of the same lines grown as xenografts in Nu/Nu mice. The gene expression profiling data resulting from hybridizing the 21 samples to Affymetrix U95A GeneChips was filtered for genes classified as present at least once across all samples.

Hierarchical clustering analysis using Spearman rank for samples and Pearson correlation for genes was used to determine the relationship between 13 different human tumor lines (Figure 4). The clustering analysis revealed that for each individual tumor line the xenograft and tissue culture profiles were more similar to each other than any other profile (with one exception). That is, tumor lines clustered together based on their genotypes rather than their growth conditions, suggesting "nature" (genotype) was more influential than "nurture" (growth conditions). This result was confirmed with MDS and principle component analysis (data not shown). The one exception from this pattern was the xenograft sample of ZR-75-1,

which did not cluster on the same node as the ZR-75-1 sample grown in tissue culture. However, subsequently we have found that our xenograft ZR-75-1 line has aberrant biological characteristics that are inconsistent with the known phenotypes of this line.

Although each tissue culture-xenograft sample did cluster together there were many gene changes between the paired samples (Table 4). When fold change values were calculated for each tissue culture xenograft pair and the number of genes with ≥2 fold-change tabulated there was a median of 425.5 genes increased in xenografts and 387 decreased. Again the ZR-75-1 sample was aberrant with many more gene changes than the other lines.

Another striking feature revealed by clustering analysis was that none of the tumor lines were particularly similar to each other. The tumor lines did not necessarily cluster based upon the tissue of origin from which each line was thought to be derived. However, a group of breast carcinoma lines that did cluster together (BT474, ZR-75-1, MDA-MB-453, MCF-7, SKBr-3 and MDA-MB-468), although MDA-MB-435 and MDA-MB-231 did not cluster with this group. The two lung carcinoma lines (H2009 and H125) did cluster closely, but MDA-MB-231 and HCT-116 (colon carcinoma) also clustered with this group. MDA-MB-435 and Colo205 clustered independently from all groups.

Selection and functional assessment of differential expressed genes in xenograft or tissue culture samples

Since genes that are co-expressed in a particular environment may provide information about the adaptive changes to the environment, we identified genes that showed a pattern of high expression in xenograft samples or tissue culture samples using Spotfire's DecisionSite calculation. This analysis identified a small number of genes that matched either pattern. There were 36 genes with increased expression in the xenograft samples relative to the tissue cultures samples and 17 genes that showed high expression in the tissue culture samples relative to the xenograft samples (Figure 5). Functional classification of these genes showed that they separated into distinct functional groups (see Table 5 and 6). Many of the genes consistently expressed in tissue culture samples encoded proteins involved in cell division, cell cycle, transcription and translation. In contrast, genes expressed in xenograft samples encoded proteins involved in extracellular matrix, cell adhesion, cell surface receptors transcription and translation.

Discussion

The first part of our study was designed to address whether gene expression patterns in xenografts varied with tumor size. No significant changes (p-value < 0.05) were found in any genes after statistical analysis of the RNA profiling data. This was a somewhat surprising result as there is evidence to suggest that tumors show size-dependent biological variation in both a clinical setting and as implanted solid tumors in rodents. It is generally assumed clinically that the larger the tumor mass the lower the likelihood of curing the patient, regardless of the treatment [17,18]. Pathological and genetic heterogeneity increase with increasing tumor cell number and contribute to the emergence of clinical drug resistance [19]. As tumors grow larger their vascular surface decreases, intercapillary distance increases, interstial pressure increases and necrotic foci develop [20]. Tumor doubling times and cell loss also increase with increasing tumor size [17].

Implanted animal tumor models most likely represent clinical end-stage disease and may not necessarily recapitulate clinical behavior seen during tumor development in humans. There are few reports on the molecular and biochemical changes that occur as implanted solid tumors increase in size. Massaad *et al.* [21] found that some drug metabolizing enzyme systems, including glutathione-S-transferases (GST) did change both activity and expression with increasing tumor size in the mouse colon adenocarcinoma Co38.

Preclinical tumor models show variability in response to treatment with a given anti-tumor agent despite being derived from a single tumor and being implanted into inbred mice [22]. This has been attributed to heterogeneity of the host or the tumor cell populations [23,24] along with tumor-to-tumor variations in perfusion and drug distribution [25]. We found there was no significant difference in gene expression between tumors of either the same size or different size as assessed in four replicate animals. If differences in tumor population, host metabolism or perfusion exist between animals implanted with the same tumors, our data suggests they are not detectable using gene expression analysis of the whole tumor.

Our results suggest that once tumor xenografts have grown to 200 mg, physiological influences on tumor transcription (such as the interaction with host stroma, nutrient supply and oxygenation) have reached steady state and do not change appreciably as the tumor grows to 1000 mg. It is also conceivable that tumors smaller and larger than the sizes investigated in this study may show gene expression changes. When a tumor is first implanted the hypoxic environment is likely to drive the expression of pro-angiogenic cytokines that will recruit new vessels to the tumor. As a tumor becomes very large (>2 g) angiogenic factors may again become highly expressed due to the inability for the existing vasculature to adequately support the large tumor mass. We have not excluded the possibility that there may be changes in protein activities

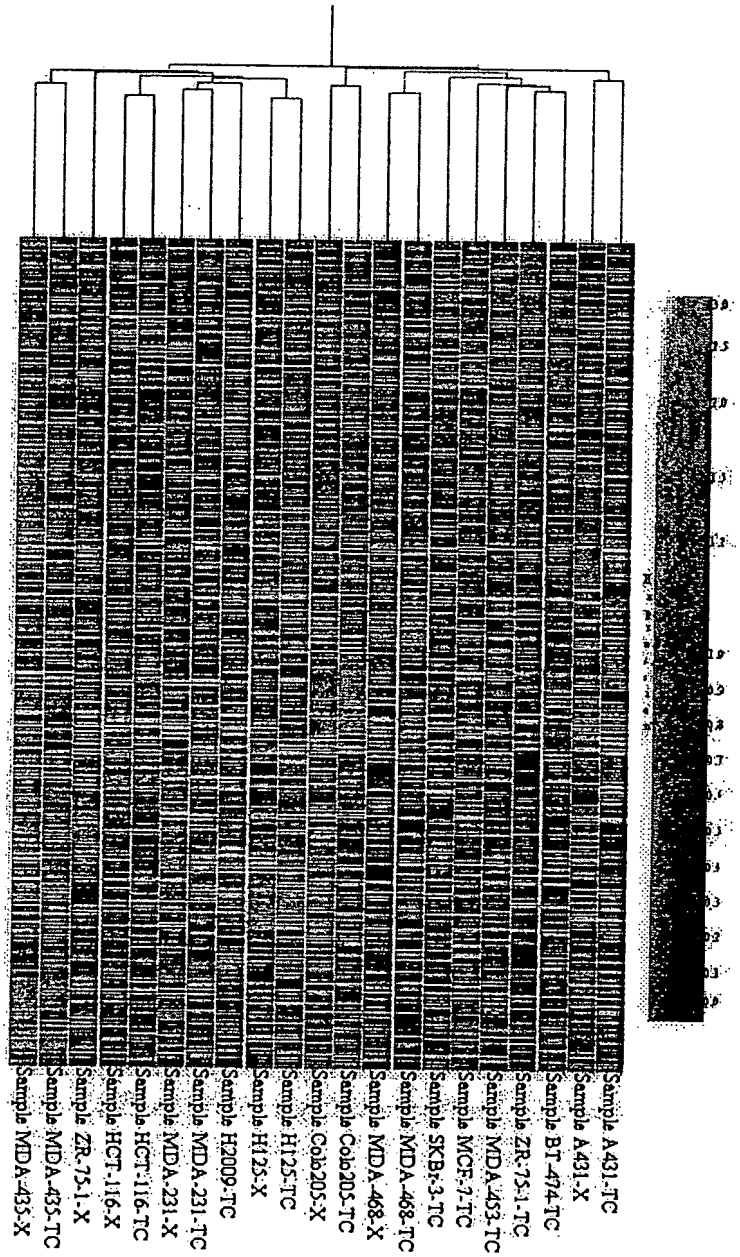

Figure 4

Hierarchical clustering analysis of 13 tumor lines grown in different environments. Hierarchical clustering analysis showing the structure within the data of the 13 tissue culture samples (suffix – TC) and 8 xenograft samples (suffix – X). Samples are displayed vertically, genes are displayed horizontally. A dendrogram of relatedness of the samples is at the top in green. For any two samples, the vertical distance from the sample roots to the first node joining them is a measure of their similarity; the shorter the distance the more similar. The color in each cell of the table represents the median adjusted expression value of each gene. The color scale used to represent the expression ratios is shown on the right, with yellow indicating increased expression relative to the median and blue decreased.

Table 2: Genes with high expression in HCT-116 tumors grown in Nu/Nu mice

Function	Affymetrix Probe Set ID	ANOVA p-value	BH p-value[1]	Log2 FC[2]	Gene Symbol	LocusLink ID	Sequence Probe Derived From	Gene Title
Translation/RNA binding/RNA splicing								
	32276_at	0.00036	0.047	-0.5	RPL32	6161	X03342	ribosomal protein L32
	32432_f_at	0.00032	0.046	-0.6	RPL15	6138	L25899	ribosomal protein L15
	33614_at	0.00028	0.046	-0.5	RPL18A	6142	X80822	ribosomal protein L18a
	33660_at	0.00039	0.048	-0.5	RPL5	6125	U14966	ribosomal protein L5
	34317_g_at	0.00029	0.046	-0.5	RPS15A	6210	W52024	ribosomal protein S15a
	34345_at	0.00043	0.049	-0.1	C20orf14	24148	AF026031	chromosome 20 open reading frame 14
	398_at	0.00029	0.046	-0.6	DDX18	8886	X98743	DEAD/H box polypeptide 18 (Myc-regulated)
	40887_g_at	0.00017	0.046	-0.5	EEF1A1	1915	L41498	eukaryotic translation elongation factor 1 alpha 1
Signal Transduction								
	35772_at	0.00029	0.046	-0.9	ARHGEF 12	23365	AB002380	Rho guanine nucleotide exchange factor (GEF) 12
	36091_at	0.00038	0.048	-0.6	SCAP2	8935	AF051323	src family associated phosphoprotein 2
	40784_at	0.00003	0.028	-0.7	PPP2R5C	5527	Z69030	protein phosphatase 2, regulatory subunit B (B56), gamma isoform
	769_s_at	0.00032	0.046	-0.3	ANXA2	302	D00017	annexin A2
	777_at	0.00010	0.039	-0.6	GDI2	2665	D13988	GDP dissociation inhibitor 2
Unknown or Other Functions								
	1160_at	0.00024	0.046	-0.3	CYC1	1537	J04444	cytochrome c-1
	1226_at	0.00010	0.039	-0.5	ADAM17	6868	U69611	a disintegrin and metalloproteinase domain 17
	31531_g_at	0.00007	0.030	-0.5	ACACB	32	U89344	acetyl-Coenzyme A carboxylase beta
	31684_at	0.00004	0.030	-0.8	ANXA2P 1	303	M62896	annexin A2 pseudogene 1
	32518_at	0.00014	0.042	-0.6	ZNF259	8882	AF019767	zinc finger protein 259
	33363_at	0.00041	0.049	-0.1	JTV1	7965	W25934	JTV1 gene
	33389_at	0.00017	0.046	-1.0	CYP51	1595	U23942	cytochrome P450, 51
	33558_at	0.00012	0.039	-0.5	TBX5	6910	Y09445	T-box 5
	33820_g_at	0.00000	0.007	-0.7	HSPA8	3312	X13794	heat shock 70 kDa protein 8
	33937_at	0.00021	0.046	-0.6			AJ011981	Homo sapiens mRNA sequence, IMAGE clone 417820
	34808_at	0.00022	0.046	-0.4			AB023216	KIAA0999 protein
	37218_at	0.00029	0.046	-0.4	BTG3	10950	D64110	BTG family, member 3
	38553_r_at	0.00025	0.046	-1.3	BAP29	55973	AI984786	B-cell receptor-associated protein BAP29
	38589_i_at	0.00042	0.049	-0.6	PTMA	5757	M14630	prothymosin, alpha (gene sequence 28)
	39167_r_at	0.00045	0.050	-1.2	SERPINH 2	872	D83174	serine (or cysteine) proteinase inhibitor, clade H, member 2
	418_at	0.00004	0.030	-0.5	MKI67	4288	X65550	antigen identified by monoclonal antibody Ki-67
	893_at	0.00003	0.028	-0.4	E2-EPF	27338	M91670	ubiquitin carrier protein
	932_i_at	0.00031	0.046	-0.8	ZNF91	7644	L11672	zinc finger protein 91

[1]p-value resulting from Benjamini and Hochberg procedure [2]log2 fold change calculated by dividing expression level from C.B-17 SCID sample by expression level of Nu/Nu sample

Table 3: Human tumor lines used in this study

Tumor	Tissue of Origin	Xenograft sample	Tissue culture sample
BT-474	Breast	No	Yes
MCF-7	Breast	No	Yes
MDA-MB-231	Breast	Yes	Yes
MDA-MB-435	Breast	Yes	Yes
MDA-MB-453	Breast	No	Yes
MDA-MB-468	Breast	Yes	Yes
SKBr-3	Breast	No	Yes
ZR-75-1	Breast	Yes	Yes
Colo205	Colon	Yes	Yes
HCT-116	Colon	Yes	Yes
H125	Lung	Yes	Yes
H2009	Lung	No	Yes
A431	Squamous cell	Yes	Yes

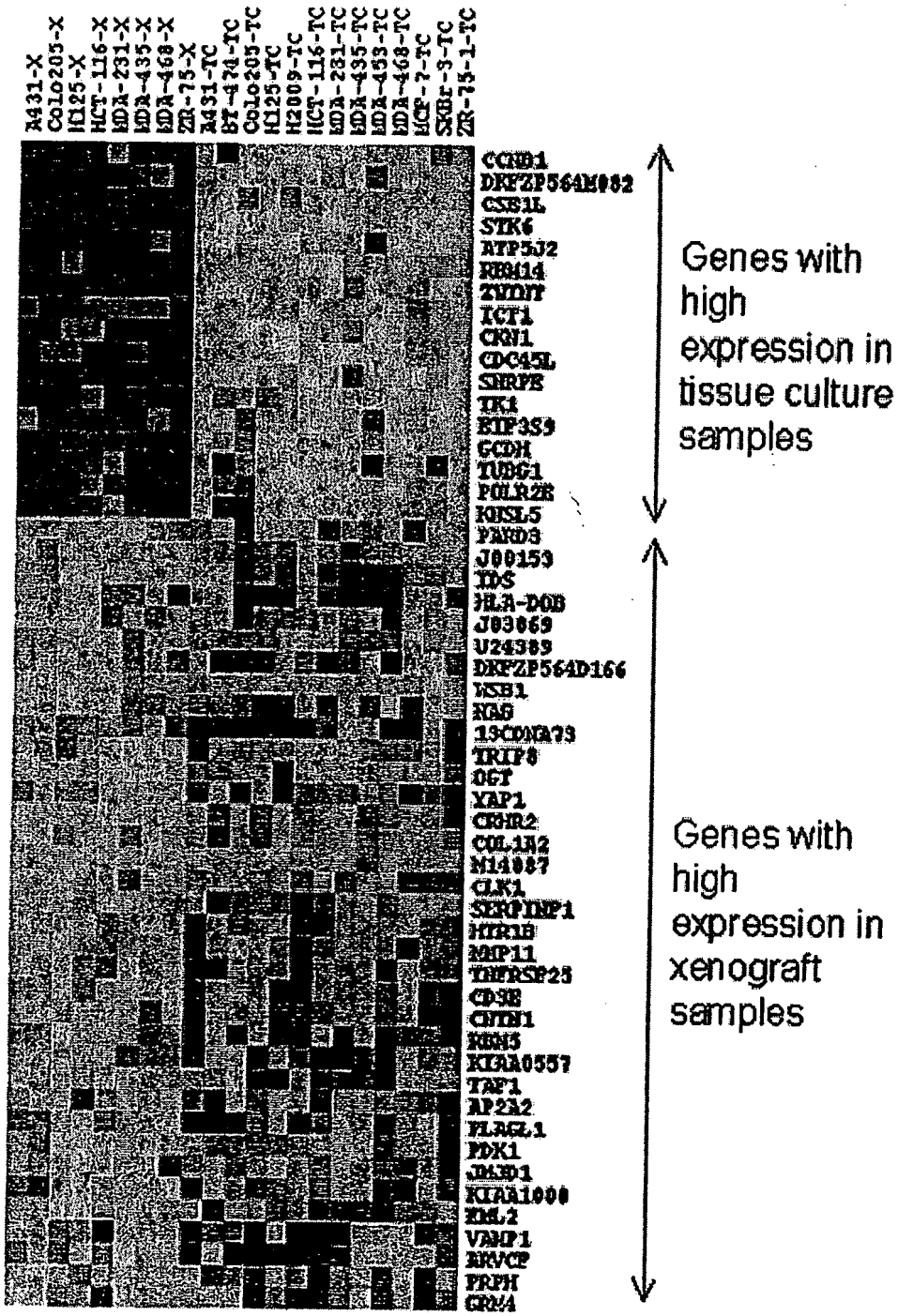

Figure 5

Heat-map plot of differential expressed genes in xenograft or tissue culture samples. Heat-map plot showing the genes selected as differentially expressed between the tissue culture and xenograft samples. The samples are displayed vertically, genes are displayed horizontally. Yellow indicates high expression while blue indicates low expression, relative to the median expression for each gene across all samples

Table 4: Number of genes with fold change values greater than or equal to 2 with tissue culture sample as the baseline

Tumor line	Number of genes with ≥2 fold increase	Number of genes with ≥2 fold decrease	Total with ≥2 fold change
A431	542	650	1192
Colo205	447	328	775
H125	335	304	639
HCT-116	351	393	744
MDA-MB-231	489	381	870
MDA-MB-435	404	519	923
MDA-MB-468	381	204	585
ZR-75-1	889	1218	2107
Median	425.5	387	822.5

or levels, or that other tumor lines may show changes as the size increases. But since the phenomenon was independently observed in two tumor lines it suggests it may be common to many other lines. The results also imply that tumors harvested within this size range without drug treatment should be very comparable. It remains possible that selective isolation and analysis of tumor regions or subpopulations by microdissection may identify expression differences.

By comparing the same tumor line grown in different mouse strains we identified 63 genes with a significant change in gene expression with biological functions ranging from cellular signaling, RNA processing and translation. Although different genes were differentially expressed in different mouse strains, particular gene functions did not associate with one strain. Therefore, the biological significance of these changes remains unclear. It should be noted that although the changes were statistically significant, in general the magnitude of the change was small. Only 12% of the significant genes displayed a change greater or equal to 2-fold. This raises the question of whether statistical significance equates to biological significance and how relevant thresholds can be determined for analyzing expression profiling data.

In the second part of the study we compared the gene expression patterns of tumor lines grown as xenografts with the same lines grown in tissue culture. We found that each xenograft – tissue culture pair was more similar to one another than any other line. This has been previously observed with RNA expression profiling of small cell lung cancer lines grown as both xenografts and tissue culture [3]. In our data, the one exception to this clustering pattern, ZR-75-1, exhibited aberrant growth characteristics; it had a rapid doubling time (2–3 days) and was not estrogen dependent as has been reported [26,27].

There was a gene expression pattern consistent with growth either in a xenograft or tissue culture environment, but remarkably it consisted of only a small number of genes. We identified 36 genes with high relative expression in xenograft samples and 17 genes with high relative expression in tissue culture samples. Their biological functions were consistent with the differences between a tissue culture sample and a xenograft sample. Genes expressed at increased levels in tissue culture samples were primarily involved in cell division, cell cycle, transcription and translation and were consistent with a greater percentage of cycling cells in the tissue culture samples, which we have previously observed with flow cytometry (data not shown). Many of the genes expressed in xenograft samples encoded for proteins involved in extracellular matrix, cell adhesion, cell surface receptors transcription and translation and suggest increased interactions with an *in vivo* stromal environment, including the development of a 3-dimensional matrix.

There are several reports in the literature of tumor line expression profiling studies demonstrating that lines derived from a common tissue of origin group together in most cases [1,4,5]. We observed that most of the breast tumor lines clustered together but the other lines generally did not show clustering patterns based upon tissue of origin. However, our study evaluated fewer samples and consisted predominantly of breast cancer tumor lines with only two representatives of non-small cell lung cancer and colon cancer.

Since each tumor line was substantially different to every other line tesed it suggests that it would be possible to identify a group of genes that could be used to distinguish

Table 5: Genes with high expression in xenograft samples

Function	Affymetrix Probe Set ID	Gene Symbol	LocusLink ID	Sequence Probe Set Derived From	Gene Title
Extracellular Matrix/Cell Adhesion					
	31574_i_at			M14087	Beta-galactoside-binding lectin, mRNA sequence
	31810_g_at	CNTN1	1272	Z21488	contactin 1
	32305_at	COL1A2	1278	J03464	collagen, type I, alpha 2
	32701_at	ARVCF	421	U51269	armadillo repeat gene deletes in velocardiofacial syndrome
	36298_at	PRPH	5630	L14565	peripherin
	38181_at	MMP11	4320	X57766	matrix metalloproteinase 11 (stromelysin 3)
	38570_at	HLA-DOB	3112	X03066	major histocompatibility complex, class II, DO beta
	38614_s_at	OGT	8473	U77413	O-linked N-acetylglucosamine (GlcNAc) transferase
Cell surface receptor					
	33950_g_at	CRHR2	1395	AF011406	corticotropin releasing hormone receptor 2
	35485_at	GRM4	2914	X80818	glutamate receptor, metabotropic 4
	35503_at	HTR1B	3351	M81590	5-hydroxytryptamine (serotonin) receptor 1B
	36277_at	CD3E	916	M23323	CD3E antigen, epsilon polypeptide (TiT3 complex)
	41190_at	TNFRSF25	8718	U83598	tumor necrosis factor receptor superfamily, member 25
Tanscription/transcription factor					
	34786_at	JMJD1	55818	AB018285	zinc finger protein
	36944_f_at	PLAGL1	5325	U72621	pleiomorphic adenoma gene-like 1
	37491_at	TAF1	6872	D90359	TAF1 RNA polymerase II, TATA box binding protein-associated factor
	38216_at	TRIP8	9323	L40411	thyroid hormone receptor interactor 8
Intracellular transport					
	32228_at	AP2A2	161	AB020706	adaptor-related protein complex 2, alpha 2 subunit
	33779_at	VAMP1	6843	AF060538	vesicle-associated membrane protein 1 (synaptobrevin 1)
Translation/RNA binding/RNA splicing					
	1556_at	RBM5	10181	U23946	RNA binding motif protein 5
	31896_at	NAG	51594	AL050281	neuroblastoma-amplified protein
Unknown or other functions					
	1155_at			J03069	
	1529_at	13CDNA73	10129	U50534	hypothetical protein CG003
	31525_s_at			J00153	
	31652_at	KIAA1000	22989	AB023217	KIAA1000 protein
	32355_at	DKFZP564 D166	26115	AL050270	putative ankyrin-repeat containing protein
	36811_at			U24389	
	37979_at	YAP1	10413	X80507	Yes-associated protein 1, 65 kDa
	39499_s_at	PARD3	56288	W25794	par-3 partitioning defective 3 homolog
	40928_at	WSB1	26118	W26496	SOCS box-containing WD protein SWiP-1
	41113_at	KIAA0557	26048	AI871396	KIAA0557 protein
	41328_s_at	EML2	24139	AL096717	echinoderm microtubule associated protein like 2
	40856_at	SERPINF1	5176	U29953	serine (or cysteine) proteinase inhibitor, clade F, member 1
	32833_at	CLK1	1195	M59287	CDC-like kinase 1
	39451_i_at	IDS	3423	AF050145	iduronate 2-sulfatase (Hunter syndrome)
	36386_at	PDK1	5163	L42450	pyruvate dehydrogenase kinase, isoenzyme 1

individual lines. Also, it has been our experience that it is possible to identify aberrant samples resulting from labeling error by expression profiling that were missed by other methods. What is less clear is whether genetic drift causes changes in RNA expression that can be identified using RNA profiling technology. Recently a report has been published showing that MCF-7 sublines were substantially different to each other both at the genetic and RNA expression levels [28] suggesting that variation within individual lines can be identified. Given this, it seems reasonable that RNA expression profiling could be used as a comprehensive methodology for identifying aberrant or incorrectly labeled samples. This would provide an additional quality control tool to standardize tumor models and the *in vivo* testing of therapeutic agents.

Conclusions

Our data suggest that the environment a tumor line is grown in can have a significant effect on gene expression even though tumor size has little or no effect for a subcutaneously grown solid tumor. Furthermore, an individual tumor line has an RNA expression pattern that clearly defines it from other lines even when grown in different environments such as tissue culture or *in vivo*. Routine RNA expression profiling of a selected set of genes could be used as a quality control tool for preclinical oncology

Table 6: Genes with high expression in tissue culture samples

Function	Affymetrix ProbeSet ID	Gene Symbol	LocusLink ID	Sequence ProbeSet Derived From	Gene Title
Cell division					
	34851_at	STK6	6790	AF011468	serine/threonine kinase 6
	33346_r_at	TUBG1	7283	M61764	tubulin, gamma 1
	35995_at	ZWINT	11130	AF067656	ZW10 interactor
	37171_at	KNSL5	9493	X67155	kinesin-like 5 (mitotic kinesin-like protein 1)
Cell cycle					
	34736_at	CCNB1	891	M25753	cyclin B1
	37458_at	CDC45L	8318	AJ223728	CDC45 cell division cycle 45-like (S. cerevisiae)
	38804_at	CSE1L	1434	AF053641	CSE1 chromosome segregation 1-like (yeast)
Translation/RNA binding/RNA splicing					
	35323_at	EIF3S9	8662	U78525	eukaryotic translation initiation factor 3, subunit 9 eta, 116 kDa
	38679_g_at	SNRPE	6635	AA733050	small nuclear ribonucleoprotein polypeptide E
	41460_at	RBM14	10432	AF080561	RNA binding motif protein 14
Transcription/transcription factor					
	39664_at	CKN1	1161	U28413	Cockayne syndrome 1 (classical)
	41332_at	POLR2E	5434	D38251	polymerase (RNA) II (DNA directed) polypeptide E, 25 kDa
Unknown or other functions					
	35715_at	DKFZP564M082	25906	AL080071	DKFZP564M082 protein
	40758_at	ICT1	3396	X81788	immature colon carcinoma transcript 1
	1749_at	GCDH	2639	AD000092	glutaryl-Coenzyme A dehydrogenase
	40134_at	ATP5J2	9551	AF047436	ATP synthase, subunit f, isoform 2
	41400_at	TK1	7083	K02581	thymidine kinase 1, soluble

studies and would allow the standardization of tumor models used worldwide.

Competing interests

None declared.

Authors' contributions

MAG carried out sample preparation, RNA isolation, data analysis and drafted the manuscript. MZM performed the MDS analysis, ANOVA and statistical analysis. NAG performed the animal procedures, sample preparation and RNA isolation. SJM directed the team who carried out the Affymetrix GeneChip hybridizations and initial data processing. EPK conceived part of the study and participated in its design. WRL conceived part of the study and participated in its design.

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Heterogeneous responses of an in vitro model of human stomach cancer to anticancer drugs.

Barranco SC, Townsend CM Jr, Quraishi MA, Burger NL, Nevill HC, Howell KH, Boerwinkle WR.

Four permanent clones of a human adenocarcinoma of the stomach and the parent line from which they were isolated were used as an in vitro model system to evaluate the effects of 8 anticancer agents on cell survival. The drugs tested were actinomycin D (Act-D), Bleomycin (Bleo), adriamycin (adria), melphalan, chlorambucil, 5 Fluorouracil (5FU), 1,2:5,6-Dianhydrogalactitol (DAG), and 1-(2-chloroethyl)-3-(4-methyl cyclohexyl-1-nitrosourea) (MeCCNU). Although the cell lines had similar growth properties, morphologies and modal chromosome numbers, the clones expressed heterogeneous survival responses to each of six drugs tested. A comparison of the doses lethal to 90% of a clonal population (LD90) for each drug indicated large differences between the most sensitive and least sensitive clones. For chlorambucil there was a 160% difference between the LD90 values of the most and least sensitive clones. For MeCCNU the difference was 200%; for adria, 230%; Bleo, 280%; 5FU, 360%; and melphalan, 600%. Despite the heterogeneity in response among the clones to these agents, no particular clone was always the most sensitive or resistant. Of particular interest was the finding that these stomach cancer clones demonstrated uniform responses to both Act-D and DAG. Since the differential drug sensitivities expressed by heterogeneous tumor populations could be a cause of treatment failure in the patient, the demonstration of uniform sensitivities to Act-D and DAG are encouraging and suggest that other anticancer drugs which produce uniform cell killing may be identified and tested. Act-D and adria were the most effective of the drugs tested when compared on a dose for dose basis. Both agents killed more than 99.9% of the parent cell line with doses below 3 micrograms/ml (1-h treatments). The cells were least sensitive to 5FU, with only 30% of the cells killed at 100 micrograms/ml. The studies reported here indicate that this human stomach cancer model can provide valuable insight into the design of clinical protocols for treatment of gastric carcinoma in man.

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Establishment and characterization of an in vitro model system for human adenocarcinoma of the stomach.

Barranco SC, Townsend CM Jr, Casartelli C, Macik BG, Burger NL, Boerwinkle WR, Gourley WK.

Ten permanent clones derived from a single biopsy specimen of an untreated human adenocarcinoma of the stomach were established and characterized in vitro. Tissue culture growth properties, doubling times, plating efficiencies, growth fractions, cell cycle phase distributions, DNA indices, modal chromosome numbers, and ploidies were determined. Growth fractions were nearly 100%, and doubling times ranged from 23 to 37 hr. The plating efficiencies were generally high for tumor cells in culture, ranging up to 70%. Modal chromosome numbers varied from 45 to 48, with a wider range of variability in about 25% of the cells studied in each clone. In addition, the parent cell line (from which the clones were isolated) was shown to grow in athymic mice and to have the same histochemical and cytological characteristics as the specimen taken from the patient. It is important to characterize human tumor cells in vitro in this detailed manner, since they serve as excellent model systems for other studies involving the heterogeneous responses to drugs and radiation. The identification of mechanisms of drug sensitivity and resistance and the testing of drug and radiation combination treatment schedules in such in vitro systems can provide valuable insight into the design of clinical protocols for treatment of stomach cancer in humans.

PMID: 6831414 [PubMed - indexed for MEDLINE]

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appl. No. : 10/725,214 Confirmation No. 4443

Applicants : Muraleedharan G. Nair, Yanjun Zhang and Shaiju Vareed

Title : METHOD FOR INHIBITING CANCER CELLS

Filed : December 1, 2003

TC/A.U. : 1655

Examiner : Michele C. Flood

Docket No. : MSU 4.1-672

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MAIL STOP AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

SUPPLEMENTAL DECLARATION UNDER 37 C.F.R. § 1.132

Dear Sir:

Muraleedharan G. Nair states as a supplement to his Declaration Under 37 CFR 1.132 dated June 23, 2006 as follows:

(1.) That Cancer Letters 194, pages 13 to 19 (2003) (Exhibit A; attached), of which he is one of the authors, clearly shows that there is a direct correlation between *in vitro* and oral *in vivo* use in suppressing multiplicity of human cancer cells of the stomach or colon with anthocyanins and cyanidin. Malvidin is a related compound to cyanidin (see Figures 1 and 2 of the application). It would be expected by one skilled in the art that there would be a similar correlation with *in vitro* and *in vivo* use of malvidin to suppress multiplicity of stomach or colon cancer cells and the adenoma of new Claims 8 to 10.

(2.) That in his opinion, the results with malvidin *in vitro* are predictive of *in vivo* activity suppressing multiplicity of cancer cells based upon his research as set forth in the Cancer Letters publication.

(3.) That the undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements

were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Muraleedharan G. Nair

Date: 9-20-06

EXHIBIT A

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Tart cherry anthocyanins inhibit tumor development in Apc^{Min} mice and reduce proliferation of human colon cancer cells☆

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Abstract

Anthocyanins, which are bioactive phytochemicals, are widely distributed in plants and especially enriched in tart cherries. Based on previous observations that tart cherry anthocyanins and their respective aglycone, cyanidin, can inhibit cyclooxygenase enzymes, we conducted experiments to test the potential of anthocyanins to inhibit intestinal tumor development in Apc^{Min} mice and growth of human colon cancer cell lines. Mice consuming the cherry diet, anthocyanins, or cyanidin had significantly fewer and smaller cecal adenomas than mice consuming the control diet or sulindac. Colonic tumor numbers and volume were not significantly influenced by treatment. Anthocyanins and cyanidin also reduced cell growth of human colon cancer cell lines HT 29 and HCT 116. The IC_{50} of anthocyanins and cyanidin was 780 and 63 μM for HT 29 cells, respectively and 285 and 85 μM for HCT 116 cells, respectively. These results suggest that tart cherry anthocyanins and cyanidin may reduce the risk of colon cancer.

Keywords: Anthocyanins; Cancer; Colon; Intestine; Mouse

1. Introduction

Tart cherries contain substantial quantities of anthocyanins in addition to other bioflavonoids [1]. Anthocyanins (Fig. 1), a member of the bioactive phytochemicals, are widely distributed in fruits, vegetables and beans, suggesting that plant-based diets can provide considerable amounts of anthocyanins [2,3]. Like the vast majority of flavonoids, anthocyanins primarily occur in plants as glycosides. Cyanidin is the major anthocyanin aglycone in tart cherries. Montmorency and Balaton™ tart cherries contain 0.40–0.80 mg/g, respectively, of anthocyanins [1]. These anthocyanins were found to function as antioxidants and cyanidin was shown to inhibit the activities of cyclooxygenase (COX) enzymes in vitro [2,4]. Several studies have demonstrated that non-

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Abbreviations: COX, cyclooxygenase; NSAIDs, non-steroidal anti-inflammatory drugs; APC, adenomatous polyposis coli; Min, multiple intestinal neoplasia; PBS, phosphate buffered saline; DMSO, dimethyl sulfoxide.

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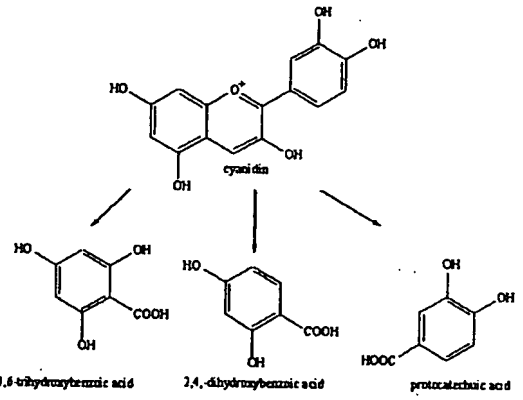

Fig. 1. Structures of the predominant tart cherry anthocyanin aglycone, cyanidin, and its major degradation products in cell culture medium.

steroidal anti-inflammatory drugs (NSAIDs) inhibit the growth of colon tumors in animal models and reduce the risk of colon cancer in humans [5,6]. In most cases, colon carcinogenesis depends on mutation of the adenomatous polyposis coli (APC) gene, which is considered a gatekeeper in the carcinogenic process [7]. Human APC gene germline mutations cause familial adenomatous polyposis, an autosomal dominantly inherited disease that predisposes affected individuals to develop numerous adenomatous polyps and, ultimately, colorectal cancer. APC gene mutations also are a frequent and early event in sporadic colon cancer. Apc^{Min} mice are a mutant mouse lineage predisposed to multiple intestinal neoplasia (Min) due to a mutation in the murine homolog of the APC gene [8]. The primary phenotype of Apc^{Min} mice is the development of multiple intestinal adenomas.

The objectives of this research were to determine the potential of tart cherry anthocyanins and cyanidin to inhibit intestinal tumor development in Apc^{Min} mice and to determine the potential of anthocyanins and cyanidin to directly inhibit the growth of human colon cancer cells.

2. Materials and methods

2.1. Animals and diets

This research was conducted with approval of the Michigan State University All-University Committee on Animal Use and Care. Apc^{Min} mice were produced by mating normal C57BL/6J ($Apc^{+/+}$) female mice with Min C57BL/6J ($Apc^{Min/+}$) male mice. Apc^{Min} progeny were identified by a polymerase chain reaction (PCR)-based assay [8] and were randomly assigned to five treatment groups ($n = 10$ per group; equal numbers of males and females) at 4–5 weeks of age and fed treatment diets for 10 weeks. The treatments were: (1) control diet (modified American Institute of Nutrition 93G diet containing 220 g/kg protein, 150 g/kg soybean oil and 50 g/kg cellulose); (2) control diet + 800 mg/l anthocyanins in the drinking water; (3) control diet + 200 mg/l cyanidin in the drinking water; (4) control diet + 200 mg/l sulindac (an NSAID) in the drinking water; and (5) modified control diet containing 200 g/kg freeze-dried pitted tart cherries. Cherries were frozen, freeze-dried and ground before they were incorporated into the diet. Anthocyanins were isolated from tart cherries and were a mixture of 3-cyanidin 2″-*O*-β-D-glucopyranosyl-6″-*O*-α-L-rhamnopyransyl-β-D-glucopyranoside [1] and 3-cyanidin 6″-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside [2] at 65 and 35%, respectively. The aglycone, cyanidin [3] (Fig. 1), was prepared from the anthocyanins [1]. Ascorbic acid (50 mg/l) was added to the drinking water of all mice to enhance the stability of anthocyanins and cyanidin in solution by lowering the pH. The level of anthocyanins and cyanidin used in this experiment were based on concentrations previously demonstrated to have antioxidant or COX-inhibitory activities in vitro. The diet containing tart cherries used in this study was not balanced to the other treatments on the basis of anthocyanin concentration, but rather represented the highest level of substitution of cherries into the control diet that could realistically be achieved without compromising the nutritional adequacy of the diet. Sulindac was included as a control in this experiment because it has consistently been shown to inhibit small intestinal adenoma development in Apc^{Min} mice.

2.2. Tumor number and size

The mice were sacrificed after 10 weeks of treatment and the numbers and sizes of adenomas in the intestinal sections were measured. The entire small intestine, cecum, and colon were removed from each mouse to determine the number and size of

adenomas. Intestinal sections were opened longitudinally, rinsed thoroughly with water, fixed overnight in 10% neutral-buffered formalin, and then stained with 0.2% methylene blue. Tumor numbers and dimensions for each intestinal segment were determined by direct counting with the aid of a dissecting microscope and measuring grid. The tumor sizes were determined by measuring the spherical (three dimensional) volume of adenomas in the cecum and colon and the average diameter of tumors in the small intestine. Tumors in the cecum and colon of Apc^{Min} mice typically are polypoid in appearance, whereas the small intestinal tumors are sessile. Spherical volumes of cecal and colonic tumors were calculated by the formula: Volume = 0.524 × (width × length × height of tumor).

Tumor numbers and diameters in the small intestine were analyzed by two-way analysis of variance (treatment, sex). For tumor numbers and volumes in cecum and colon, data were transformed to ranks and then ranks were analyzed by two-way analysis of variance. When significant treatment effects were detected ($P < 0.05$), treatment means were compared using the Least Significant Difference method. Six mice (one from the control group, two from the anthocyanin group, and three from the cyanidin group) were excluded from the final statistical analysis because they did not develop intestinal tumors. Confirmatory PCR analysis conducted at the end of the experiment indicated that these animals did not carry the APC^{Min} gene mutation.

2.3. Cell culture and growth assays

The human colorectal cancer cell lines HCT 116 and HT 29 (American Type Culture Collection) were cultivated in McCoy's 5A media supplemented with 10% fetal bovine serum. Cells were harvested for growth assays when they had reached 50–80% confluence by trypsin:ethylenediaminetetraacetic acid (EDTA) treatment and counted using a hemacytometer. Cells were then seeded at 15 000 cells/well in 24-well tissue culture plates. Plates were incubated overnight at 37°C and 5% CO_2 to allow cells to attach and begin proliferating.

At the beginning of treatments, the media was gently aspirated from each of the wells, which were then rinsed with phosphate buffered saline. One ml of treatment media was added to each well ($n = 8–12$ wells per treatment level per cell line) and plates were incubated for 72 h. Treatment media was McCoy's 5A media supplemented with 10% fetal bovine serum and containing the respective concentrations of anthocyanins (0–1000 μM) or cyanidin (0–250 μM). Anthocyanins were dissolved in distilled water before addition to the treatment media, whereas cyanidin was dissolved in dimethyl sulfoxide (DMSO) before addition. When used, the DMSO concentration was equalized for all treatment media and never exceeded 0.1% (v/v) of the final treatment media.

Total cell numbers in each well were quantified after 72 h of incubation in treatment media. Cell numbers were calculated based on total DNA content in each well using a procedure that quantifies DNA based on fluorescence of bound Hoescht 33258 [9]. Fluorescence was measured by a Cytofluor II fluorimeter (Applied Biosystems; Foster City, CA) using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Fluorescence readings were converted to DNA by comparison to standard solutions of Salmon testis DNA (Sigma Chemical Company; St. Louis, MO). The cell

Table 1
Influence of anthocyanins, cyanidin, tart cherries and sulindac on cecal adenoma number and volume in Apc^{Min} mice (SEM = standard error of the mean)[a]

Treatment	Adenomas/mouse	SEM	Adenoma volume/mouse (mm^3)	SEM
Control	2.3[a]	0.6	3.0[a]	0.8
Anthocyanins	0.6[b]	0.6	0.7[b]	0.9
Cyanidin	0.6[b]	0.6	0.6[b]	1.0
Cherries	0.6[b]	0.5	1.8[b]	0.8
Sulindac	4.0[a]	0.5	4.0[a]	0.8

[a] [a,b]Means in the same column not sharing a common superscript a different ($P < 0.05$).

numbers in each well were calculated by converting the quantity of DNA in each well by the amount of DNA present in each cell (determined experimentally) for HCT 116 and HT 29 cells.

Cell numbers observed in each well after 72 h of growth were corrected for initial cell number (determined at the time treatment media was added). These data were then subjected to multiple regression analysis using the general linear models procedure of SAS (Version 8.1, SAS Institute, Inc., Cary, NC, USA) to develop least-squares polynomial equations describing the influence of anthocyanins or cyanidin concentration on cell number. These equations were then used to iteratively calculate the concentration of anthocyanins or cyanidin required to cause a 50% reduction (IC_{50}) in growth (cell number) for each cell line.

3. Results

Final body weights of mice were significantly influenced by treatment and averaged 22.8, 24.1, 21.3, 19.7, and 25.5 g for mice consuming control diet, anthocyanins, cyanidin, tart cherries, and sulindac, respectively. Final body weights for mice consuming anthocyanins and sulindac were greater ($P < 0.05$) than for mice consuming tart cherries.

Treatments had differential effects on tumor incidence and burden in the various sections of the intestinal tract. Mice consuming anthocyanins, cyanidin, or tart cherries had fewer ($P < 0.05$) adenomas in the cecum than mice consuming the control diet or sulindac (Table 1). The total burden (volume) of cecal adenomas was less ($P < 0.05$) in mice consuming anthocyanins, cyanidin or tart cherries when compared to mice consuming the control diet or sulindac (Table 1). Colonic adenoma number was not significantly influenced by treatment (Table 2). Although mice that consumed tart cherries had the greatest adenoma burden in the colon (8.4 mm^3), this was not statistically greater than that observed in mice consuming the other treatments (Table 2).

Tumor multiplicity in the small intestine was not significantly influenced by treatment and averaged 48 tumors per mouse (Table 3). Mice that consumed sulindac had the smallest number of small intestinal adenomas (28 per mouse), but this was not statistically different than small intestinal tumor numbers observed for the other treatments. The average size of small intestinal adenomas (Table 3) was increased ($P < 0.05$) by feeding tart cherries and reduced ($P < 0.05$) by sulindac relative to that observed in mice consuming the control diet, anthocyanins, or cyanidin.

Treatment with anthocyanins (Fig. 2) or cyanidin (Fig. 3) caused a dose-dependent reduction in cell numbers for both HCT 116 and HT 29 cells. Neither anthocyanins nor cyanidin caused cytotoxicity even at the highest concentrations tested, as indicated by little or no dead cells. Cyanidin was far more effective in inhibiting the growth of these cancer cell lines than anthocyanins. The observed IC_{50} values for cyanidin were 85 and 63 μM for HCT 116 and HT 29 cells, respectively, whereas those for anthocyanins were 260 and 585 μM for HCT 116 and HT 29 cells, respectively.

4. Discussion

Our interest in testing the potential of tart cherry anthocyanins and cyanidin to inhibit tumor development in Apc^{Min} mice stemmed from the observation that these compounds inhibit the activities of COX

Table 2
Influence of anthocyanins, cyanidin, tart cherries and sulindac on colon adenoma number and volume in Apc^{Min} mice (SEM = standard error of the mean)

Treatment	Adenomas/mouse	SEM	Adenoma volume/mouse (mm^3)	SEM
Control	3.7	0.7	2.4	2.0
Anthocyanins	3.1	0.7	4.0	2.1
Cyanidin	3.7	0.8	3.5	2.3
Cherries	3.3	0.6	8.4	1.9
Sulindac	5.3	0.6	3.3	1.9

Table 3
Influence of anthocyanins, cyanidin, tart cherries and sulindac on small intestinal adenoma number and diameter in Apc^{Min} mice (SEM = standard error of the mean)[a]

Treatment	Adenomas/mouse	SEM	Adenoma volume/mouse (mm^3)	SEM
Control	58	13	1.39[a]	0.10
Anthocyanins	76	13	1.34[a]	0.10
Cyanidin	54	16	1.25[a]	0.11
Cherries	42	12	1.66[b]	0.10
Sulindac	28	12	0.93[c]	0.09

[a] [a,b,c]Means in the same column not sharing a common superscript a different ($P < 0.05$).

enzymes [4]. Other studies have demonstrated that sulindac (and other NSAIDs) reduce small intestinal tumor multiplicity and size in Apc^{Min} mice [10–12]. In this study, we found that anthocyanins, cyanidin, and tart cherries (presumably as a source of anthocyanins) all significantly reduced the number and burden of tumors in the cecum of Apc^{Min} mice. Conversely, sulindac did not influence tumor development in the cecum. None of the treatments tested influenced the numbers of tumors in the small intestine or the numbers or burden of tumors in the colon. Our inability to detect a significant reduction in small intestinal adenoma number by sulindac was likely due to a number of factors, including the relatively small numbers of mice per treatment group and large variations among individual mice in adenoma development. The dose of sulindac and duration of tumor promotion allowed in this experiment may also have contributed to the limited effect of sulindac on small intestinal adenoma number. The lack of effect of anthocyanins or cyanidin on colonic tumor development may be a consequence of their metabolism by intestinal bacteria or their spontaneous degradation in the cecal and colonic environment.

The average size of small intestinal tumors was smaller in mice consuming sulindac and larger in mice consuming tart cherries when compared to that in mice consuming the control diet, anthocyanins or cyanidin. Progression in the size of small intestinal tumors is highly correlated with morbidity in Apc^{Min} mice. We have observed that significant morbidity and weight loss occurs when small intestinal tumors reach an average size of 1.5 mm. At this stage, the tumors tend to hemorrhage and may perforate the small intestine. The observed differences in final body weight in this experiment are a consequence of these differences in small intestinal tumor promotion. It is well documented that sulindac and other NSAIDs consistently reduce the size of small intestinal tumors in Apc^{Min} mice [10–12]. The mechanism for the larger small intestinal tumor size in mice consuming tart cherries is not known, but merits further investigation.

The effects of the treatments on tumor development were not consistent throughout the intestinal

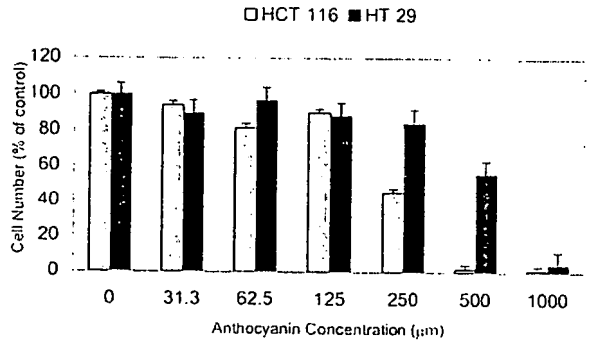

Fig. 2. Influence of anthocyanins on growth of human colon cancer cells. Gray bars, HCT 116 cells; Black bars, HT 29 cells. Error bars = standard error of the mean.

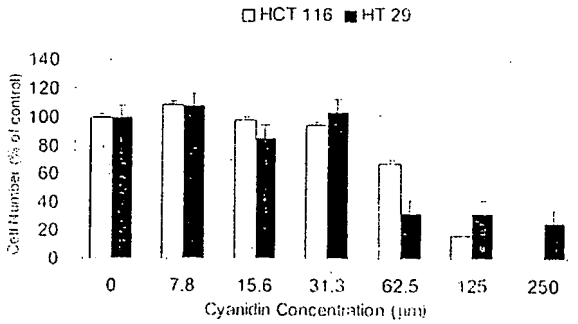

Fig. 3. Influence of cyanidin on growth of human colon cancer cells. Gray bars, HCT 116 cells; Black bars, HT 29 cells. Error bars = standard error of the mean.

tract. Sulindac and other NSAIDs typically reduce small intestinal tumor development in Apc^{Min} mice, but often have little effect on tumor development in the cecum and colon [13]. Mice consuming anthocyanins, cyanidin and tart cherries had fewer and smaller tumors in the cecum, but these compounds did not afford any protection to the small intestine. These results indicate that although anthocyanins and cyanidin inhibit the activities of COX enzymes in vitro [2,4], they likely do not influence intestinal tumor development in Apc^{Min} mice through a pathway involving COX inhibition. Meiers et al. [14] demonstrated that the anthocyanins cyanidin and delphinidin are potent inhibitors of the epidermal growth factor receptor kinase. Administration of inhibitors to the epidermal growth factor receptor kinase also has been shown to reduce intestinal adenoma development in Apc^{Min} mice [15]. We currently are investigating other potential mechanisms whereby anthocyanins and cyanidin influence intestinal tumor development.

Both anthocyanins and cyanidin inhibited the growth of the colon cancer cell lines HT-29 and HCT 116, although cyanidin was much more effective. Our observations are in agreement with those of Meiers et al. [14], who observed IC_{50} values 73 and 42 μM when cyanidin was administered to LXFL529L large cell lung tumor cells and A431 human vulva carcinoma cells, respectively. Kamei et al. [16] also demonstrated that a crude anthocyanin fraction prepared from red wine was an effective inhibitor of the growth of HCT-15 human colon tumor cells in vitro. We have identified three degradation products from anthocyanins and cyanidin in cell culture medium. These were protocatechuic acid, 2,4-dihydroxybenzoic, and 2,4,6-trihydroxybenzoic acids [3]. In addition, we have detected trace quantities of cyanidin-3-glucoside and cyanidin in culture medium after 72 h of cell growth in anthocyanin treatments. We have evaluated the potential of these degradation products to inhibit the growth of HCT 116 and HT 29 cells. None of the degradation compounds assayed demonstrated any inhibition of cell growth at concentrations ranging up to 250 μM [3].

Anthocyanins and cyanidin are unstable at pH 7.0 and spontaneously degrade to chalcone and benzoic acid derivatives. The red cyanidin cation is stable at pH <3, but deprotonates and produces ketoquinonoidal bases and finally to an ionized quinonoid base at pH >7 [3]. At pH 3–6, the cyanidin cation forms a carbinol pseudobase or chalcone pseudobase [3].

Anthocyanins are highly water-soluble and considered to be structurally similar to a number of strong DNA intercalators [17]. Both DNA and RNA act as strong copigments for anthocyanins [18]. Also, anthocyanins protect DNA against oxidative damage [19]. Under in vivo and cell culture conditions, both anthocyanins and cyanidin potentially form corresponding pseudobases due to pH variations. These pseudobases are transition compounds and may be stable in vivo as protein bound complexes. Given these results, we predict that the anthocyanins, the aglycone cyanidin, or its varying pseudobases directly suppress cell growth and subsequent tumor development.

We believe that our results are the first to demonstrate that anthocyanins and cyanidin have the potential to directly interfere with intestinal tumor development. Hagiwara et al. [20] demonstrated that anthocyanins in purple corn color reduced the promotion of colon tumors caused by 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in rats initiated with dimethyl hydrazine. However, they did not test the potential of purple corn color anthocyanins to directly suppress tumor development [20].

Although we did not directly measure intake of anthocyanins or cyanidin in this experiment, we can tentatively compare the doses administered to the mice to a comparable human dose. If we assume that the mice consumed 8 ml of water each day and also assume that there was no degradation of anthocyanins or cyanidin, then the consumed dose would be 6.4 or 1.6 mg of anthocyanins or cyanidin per day, respectively, for the mice consuming those treatments. If we assume an average weight of 25 g for the mice and 70 kg for an adult human, then the comparative human doses (on the basis of $kg^{0.75}$) would be 2400 or 600 mg of anthocyanins or cyanidin, respectively. It is very unlikely that these doses could be achieved in a typical human diet without supplementation. It should also be noted that these calculations represent very crude comparisons and do not consider potential absorption, metabolism or degradation of the compounds.

In summary, we have demonstrated that tart cherry anthocyanins and their aglycone cyanidin significantly reduced tumor development in the cecum of

Apc^{Min} mice. These compounds also directly inhibited the growth of human colon cancer cells in vitro, with the aglycone cyanidin being far more effective than the anthocyanin glycosides. Benzoic acid derivatives yielded from the degradation of anthocyanins and cyanidin have no influence on colon cancer cell growth. Taken together, these results suggest that and cyanidin or its corresponding pseudobase is directly inhibiting tumor development in the cecum of Apc^{Min} mice. Anthocyanins also are effective presumably due to their deglycosylation to cyanidin by cecal bacteria. The lack of a clear suppression of tumor development in the colon probably is due to further degradation of the cyanidin molecule by elevated pH in the intestinal lumen and bacterial metabolism.

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